Substrates specificity of tannase from *Streptomyces svieus* and *Lactobacillus plantarum*

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**Abstract**

Tannases can catalyze the hydrolysis of galloyl ester and depside bonds of hydrolysable tannins to release gallic acid and glucose, but tannases from different species have different substrate specificities. Our prior studies found that tannase from *Lactobacillus plantarum* (LP-tan) performed a higher esterase activity, while the tannase from *Streptomyces svieus* (SS-tan) performed a higher depsidase activity; but the molecular mechanism is not elucidated. Based on the crystal structure of LP-tan and the amino acid sequences alignment between LP-tan and SS-tan, we found that the sandwich structure formed by Ile206-substrate-Pro356 in LP-tan was replaced with Ile253-substrate-Gly384 in SS-tan, and the flap domain (amino acids: 225–247) formed in LP-tan was missed in SS-tan, while a flap-like domain (amino acids: 93–143) was found in SS-tan. In this study, we investigated the functional role of sandwich structure and the flap (flap-like) domain in the substrate specificity of tannase. Site-directed mutagenesis was used to disrupt the sandwich structure in LP-tan (P356G) and rebuilt it in SS-tan (G384P). The flap in LP-tan and the flap-like domain in SS-tan were deleted to construct the new variants. The activity assay results showed that the sandwich and the flap domain can help to catalyze the ester bonds, while the flap-like domain in SS-tan mainly worked on the depside bonds. Enzymatic characterization and kinetics data showed that the sandwich and the flap domain can help to catalyze the ester bonds, while the flap-like domain in SS-tan may worked on the depside bonds.

**Keywords:** Tannase, Ester and depside bonds, Kinetics, Substrate specificity

**Introduction**

Tannins, the fourth abundant plant constituent, existing as water soluble, poly-phenolic compounds is widely distributed in plant kingdom, especially in roots, leaves, fruits, and seeds. Tannins are toxic to many fungi, bacteria, and viruses (Aguiar et al. 2007). However, many microorganisms have developed the ability to grow in the presence of tannins through the induction of secreted enzymes that utilize these compounds as carbon and energy sources (Aguiar et al. 2007). These enzymes are known as tannin acyl hydrolases, including tannin acyl hydrolases (EC 3.1.1.20), commonly referred as tannases. They can catalyze the hydrolysis of ester and depside bonds of hydrolysable tannins to release gallic acid and glucose (Lopes et al. 2018; Rodríguez-Durán et al. 2011). Gallic acid was found to exert an anticancer effect against a variety of cancer cells (Tsai et al. 2018). Tannases also have application potential in the clarification of wine and soft drinks as well as de-tannification of food and animal feed for nutritional improvement (Chamorro et al. 2017; Martins et al. 2016; Li et al. 2018).

At present, the production of secreted tannase for industrial applications involves the utility of either crude or semi-purified enzyme prepared from submerged or solid-state cultures of *Aspergillus niger* or *Aspergillus oryzae* fermented in the presence of tannic acid (Aguiar et al. 2007; Varadharajan et al. 2017; Wu et al. 2018). However, low yield and purity, batch variability and a poor understanding of catalytic mechanism of tannase...
have limited its utility. In the post genomic era, with the identification of new and novel tannase genes from a range of different species, potential opportunities are available to engineer enzymes with high productivity, purity and activities (Curiel et al. 2009; Iwamoto et al. 2008; Noguchi et al. 2007; Sharma and John 2011; Wu et al. 2013, 2015).

In previous works (Ren et al. 2013), we reported the first 3D crystal structure of LP-tan (PDB no: 4U1, 4JC, 4JD, 4JG, 4JH, 4Jo, 4Ji, 4JK). The structure revealed the spatial details of the protein molecule, including its catalytic and substrate binding sites, and reaction mechanism of the tannase. From the complex structures of LP-tan, we found that the esterase and depa-sidase activities shared the same active center and catalytic mechanisms (Ren et al. 2013). However, LP-tan showed a higher esterase activity and substrate specificity. In another study, we successfully codon-optimized and chemically synthesized the SS-tan encoding gene from *Streptomyces sviceus* and cloned it into a recombinant prokaryotic heterologous expression system for high-yield tannase production (Wu et al. 2015). Compared to the LP-tan, the SS-tan showed a higher depa-sidase activity and a lower esterase activity. In order to further investigate the substrate specificity of these two tannases, the amino acid sequences alignment between SS-tan and LP-tan were performed, the results only showed 35% sequence similarity, but all the substrate binding and catalytic traid were conserved.

In this study, our aim is to investigate the substrates specificity of LP-tan and SS-tan. Based on the structures and biochemical data of LP-tan, we have proposed the substrate specificity between these two tannases, which maybe benefit the further industrial applications and modifications of tannase.

### Materials and methods

#### Protein expression and purification

Cloning, expression, and purification of tannases from *Lactobacillus plantarum* (GeneBank: AB379685.1) and *S. sviceus* (GeneBank: LK985323.1) have been described elsewhere (Wu et al. 2013, 2015). In brief, the tannase gene was inserted into a C-terminal hexa-histidine tagged protein expression vector pET-43b, and the recombinant vector was transformed into *E. coli* BL21-DE3 cells (Life Technologies, USA) for protein expression. The cells were grown at 37 °C, 200 rpm/min in 2YT medium until a cell density of 1.0 (OD600nm) reached. Protein expression was induced with the addition of 0.5 mM IPTG at 20 °C, 200 rpm/min, for 20 h. Then the cells were harvested and lysed. The lysate was centrifuged at 20,000 rpm/min for 30 min at 4 °C, and the supernatant was loaded onto a 5 ml HisTrap column (GE Healthcare) equilibrated with the loading buffer containing 20 mM Tris–HCl, 150 mM NaCl, 10 mM imidazole, pH 8.0. The column was subsequently washed and eluted with a similar buffer containing 30 and 300 mM imidazole, respectively. The collected protein was further purified by a gel filtration column (HiLoad 16/60 Superdex 200, GE Healthcare) equilibrated with 20 mM Tris–HCl, 150 mM NaCl, pH 8.0. Purity of the protein was monitored by 12% SDS-PAGE under reducing conditions.

#### Site-directed mutagenesis

Site-directed mutagenesis was performed using Quick-Change Lightning Site-directed Mutagenesis Kit (Agilent Technologies) with PCR method. Plasmid pET-43b with LP-tan gene and SS-tan gene were used as templates (primer sequences were listed in Table 1). The single point mutated proteins were expressed and purified with the same procedures as the wild-type protein.

### New variants construct

In order to investigate the substrate specificity of tannases, two new variants were developed. The variants without the flap domain (amino acid 225–247) in LP-tan and the flap-like domain (amino acid 93–144) in SS-tan were chemically synthesized. The nucleotide sequence GGAGGATCC (amino acids sequence: Gly–Gly-Ser) was used as the linker to replace the flap and flap-like domain. The variants were expressed and purified as mentioned previously.

#### Table 1 The primer sequences used in this study

| Mutated site | Primer sequences |
|--------------|------------------|
| SS-tan S210A | F: GTCCTCTCACGATACCCTGCGGGTGCCG |
|              | R: AGCGGTCCACGACGGCAATACCTTCAGG |
| SS-tan K371A | F: GTCGCTGCTACCGCTGTCGGCGAC |
|              | R: AGCGGTCCACGACGGCAATACCTTCAGG |
| SS-tan E385A | F: GACCGCTGACCGCTGTCGGCGAC |
|              | R: AGCGGTCCACGACGGCAATACCTTCAGG |
| SS-tan D453A | F: CGTCTGCTACCGCTGTCGGCGAC |
|              | R: AGCGGTCCACGACGGCAATACCTTCAGG |
| SS-tan D455A | F: GTGACCGCTGACCGCTGTCGGCGAC |
|              | R: AGCGGTCCACGACGGCAATACCTTCAGG |
| SS-tan H485A | F: TACCTGGACCGCTGTCGGCGAC |
|              | R: AGCGGTCCACGACGGCAATACCTTCAGG |
| SS-tan G384P | F: CTTTGCAGCTGTGATACCCAGAAACCAATG |
|              | R: CCAGACACCGGTGTTTCTGTCGGACGAC |
| LP-tan P356G | F: CCAGTCAGCTGATACCCAGAAACCAATG |
|              | R: TCGCAGAATATATCTTACTACCATCGCAATTCAGG |

The mutated nucleotide sites are indicated by bold italics.
Enzyme activity assay
Rhodamine reacts only with gallic acid but not with galloyl esters or other phenolics. So the tannase activity was assayed by a method based on chromogen formation between gallic acid and rhodanine (Curiel et al. 2009). The single point mutants and variants were assayed by a method based on chromogen formation between gallic acid and rhodanine. In brief, a standard curve using gallic acid concentrations ranging from 0.125 to 1 mM was prepared. Activities of recombinant tannase were measured using 25 mM methyl gallate (Sigma, USA) and 3 mM tannic acid (Sigma, USA) as substrates.

The reaction conditions with variants were optimized regarding temperature and pH. Activities of recombinant tannase were measured at pH 8.0, temperature ranged from 10 to 70 °C to determine the optimal temperature. The optimum pH value for enzymatic activity was determined at 37 °C by studying its pH-dependence within the pH range from 3 to 10. All the activity assays were performed at the optimum temperature and pH. One unit of activity was defined as the amount of enzyme required to release 1 μM of gallic acid per minute under standard reaction conditions.

Kinetics analysis
The substrates methyl gallate (0.1–5 mM) and tannic acid (0.005–0.04 mM) were incubated with appropriate amount of enzyme to calculate the kinetic parameters. The amount of gallic acid which was formed by the catalysis of tannase was calibrated using the absorbance at 520 nm. Kinetic parameters were obtained according to the Line-weaver and Burk (double reciprocal) method.

Results
Amino acid sequence alignment and analysis
The amino acid sequences alignment only showed 35% sequence similarity between SS-tan and LP-tan, but the substrate binding and catalytic triad were conserved (Fig. 1). Based on the structure analysis of LP-tan, we found that the sandwich structure formed by Ile206-Pro356 in LP-tan was replaced with Ile253-Gly384 in SS-tan, and the flap domain formed in LP-tan was missed in SS-tan, while an additional flap-like sequence (93–148) was found in the SS-tan.

Enzymatic characterization of the variants and mutants
The single point mutants and the variants were well expressed in E. coli BL21-DE3 cells and expressed at high levels. Including the C-terminal hexa-histidine tag, SDS-page showed that SS-tan variant showed a molecular mass of about 51 kDa, and LP-tan variant showed a molecular mass of about 49 kDa (Fig. 2a). SS-tan variant and LP-tan variant also displayed maximum activity at pH 8.0, and the optimum temperature for SS-tan variant was 50 °C, LP-variant was 30 °C, which is similar with the wild type (Fig. 3).

The sandwich structure was dismissed or rebuilt by site-directed mutagenesis in LP-tan and SS-tan. The single point mutant LP-tan P356G only had 48.2% esterase activity left comparing with LP-tan, while the single point mutant LP-tan P356G had a higher depsidase activity (131.7%). Comparing with SS-tan, SS-tan G384P had a higher esterase activity (138.1%) and a lower depsidase activity (61.1%) (Table 2). The SS-tan variant showed a lower (55.3%) depsidase activity and a lower (91.2%) esterase activity comparing with the wild type. In addition, the variant from LP-tan had a higher (244.7%) depsidase activity and a lower (67.6%) esterase activity (Table 3).

Kinetic analysis on substrate preference
The $k_{cat}$ and $K_m$ value were always used to compare the substrate specificity of enzymes. SS-tan showed a higher substrate affinity (lower $K_m$ value) and catalytic efficiency (high $k_{cat}/K_m$ value) for depside bonds than ester bonds, while the LP-tan showed a higher substrate affinity and catalytic efficiency for ester bonds than depside bonds (Tables 2, 3).

The $K_m$ values of LP-tan P356G for methyl gallate and tannic acid was about twofold higher and 1.8-fold smaller than the corresponding $K_m$ values of LP-tan, respectively. Compared with the wild type, LP-tan P356G had a smaller $k_{cat}/K_m$ value (about 2.4-fold) when methyl gallate was used as the substrate and a higher $k_{cat}/K_m$ value (about 1.4-fold) when tannic acid was used as the substrate. In opposite, the $K_m$ values of SS-tan G384P for methyl gallate and tannic acid were about 1.4-fold smaller and twofold higher than those values of the wild type, respectively (Table 2). When methyl gallate was used as the substrate, the corresponding $k_{cat}/K_m$ value of SS-tan G384P was about 1.4-fold higher than SS-tan.

While the corresponding $k_{cat}/K_m$ value of SS-tan G384P for tannic acid was about 1.6-fold smaller than SS-tan. Therefore, the sandwich structure could help to bind and catalyse the hydrolysis of ester bond.

Compared with SS-tan, the SS-tan variants showed higher $K_m$ value and lower $k_{cat}/K_m$ value (about 1.6-fold) when using tannic acid as the substrate, but the $K_m$ and $k_{cat}/K_m$ values for methyl gallate had no significant changes (only about 1.1 fold) (Table 3). The $K_m$ value of LP-tan variants for tannic acid and methyl gallate was about 2.4-fold smaller and 1.7-fold higher than the corresponding $K_m$ values of LP-tan, respectively. The $k_{cat}/K_m$ value (about 1.2-fold) was always higher than the corresponding $k_{cat}/K_m$ value of the wild type.
values of LP-tan variants for methyl gallate and tannic acid were about 1.7-fold lower and 2.6-fold higher than the wild type, respectively (Table 3).

**Discussion**

Tannase can catalyse the hydrolysis of ester and depside bond in hydrolysable tannins to release glucose and gallic acid. Thus, tannase was crucial in the industries of foods, drinks and pharmaceutics (Chamorro et al. 2017; Lekha and Lonsane 1997; Li et al. 2018; Martins et al. 2016). In the past decades, both the high-yield production of tannase and the high activity conservation were considered as the research priorities. Since the discovery of tannase, its esterase and depsidase activities have been in debate for a long time (Haslam and Stangroom 1966). However, only few reports described the catalytic mechanism and substrate specificity of tannase. In the previous works, our group reported the crystal structure of LP-tan, which was the first tannase 3D structure (Ren et al. 2013). Based on the apo and complexes structures of LP-tan, the hydrolysis mechanism of tannase was explained. Mutagenesis studies demonstrated that the esterase and depsidase activities of LP-tan shared the same catalytic site. When LP-tan binding the substrates, two hydrogen-bond binding networks were observed, the first network was formed between the amino acids G77, A164, S163, H451 and the carboxyl group of galloyl unit of the substrates; the other hydrogen-bonding network was formed...
between amino acids E357, K343, P421 and the hydroxyl groups of the galloyl unit of the substrates (Ren et al. 2013). The hydrogen-bonding networks could help to bind the substrates and were necessary for the catalytic.

According to the amino acid alignment, the substrate binding and catalytic triad were conserved in SS-tan (Fig. 1). Site-directed mutagenesis of each residue in the catalytic center (Ser210A) and hydrogen-bonding

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**Fig. 2**  
a The purified tannases were analyzed with 12% SDS-PAGE. M: molecular mass of standards; (1) LP-tan; (2) LP-tan variant; (3) SS-tan; (4) SS-tan variant.  
b Activities of site-directed mutagenesis of SS-tan relative to wild-type of SS-tan. Methyl gallate was used as the substrate and each measurement was performed in quintuplicate.

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**Fig. 3**  
Effects of pH and temperature on the activities of LP-tan variant and SS-tan variant. In all the cases the observed maximum activity was defined as 100% and each experiments were performed in quintuplicate. Methyl gallate was used as the substrate.
networks (K371A, E385A, D453A, D455A, H485A) resulted in an almost complete abolished in esterase and depsidase activities of SS-tan (Fig. 2b). Therefore, the esterase and depsidase activities of SS-tan also shared the same catalytic site and mechanism. However, these two enzymes possess different substrate specificity; LP-tan had a higher esterase activity while the SS-tan had a higher depsidase activity (Table 2). Based on the complex structures of LP-tan, the galloyl unit of the substrate was almost buried by the amino acids Gly77 and Pro356, which then combined with the amino acid His206 to form the sandwich-like structure to stabilize the bond of galloyl unit. However, the sandwich structure was not found in the SS-tan, because the Pro356 used to form the sandwich structure in LP-tan was replaced with Gly384 at the same position in SS-tan.

To investigate the function of the sandwich structure in the substrate specificity, we mutated Pro356 to Gly356 (LP-tan P356G) in LP-tan and mutated Gly384 to Pro384 in SS-tan (SS-tan G384P). Compared with the wild type, LP-tan P356G showed significantly higher substrate affinity and catalytic efficiency for depside bonds, while SS-tan G384P showed a higher affinity and catalytic efficiency for esterase bonds (Tables 2, 3). The galloyl unit was the only clear part that could be observed from the electron density map in the complex structure of LP-tan, which means that the substrates had high complexity and flexibility. Therefore, the sandwich structure of tannase might have dual functions. When tannase digested small substrates, like methyl gallate (ester bond), hydrogen-bonding network was formed to bind the substrates, and the amino acid proline in sandwich structure could help to stabilize the binding the galloyl unit of substrates. When tannase digested large substrates, like tannic acid (depside bond), hydrogen-bonding network also formed in the activity center with the galloyl unit. However, due to the complexity and flexibility of the substrates, the proline in the sandwich structure might form hydrophobic reactions with the other galloyl units to prevent the substrates from binding to the activity center.

Table 2 Activities of LP-tan, SS-tan, LP-tan P356G, SS-tan G384P and the variants

|          | Methyl gallate (esterase, U/mg) | Tannic acid (depsidase, U/mg) |
|----------|---------------------------------|-------------------------------|
|          | Km (mM) | kcat (s⁻¹) | kcat/Km (s⁻¹mM⁻¹) | Km (mM) | kcat (s⁻¹) | kcat/Km (s⁻¹mM⁻¹) |
| LP-tan   | 0.67 ± 0.15 | 99.4 ± 11.4 | 158.4 ± 35.1 | 1.23 ± 0.4 | 62.6 ± 13.5 | 51.9 ± 16.0 |
| LP-tan P356G | 1.3 ± 0.29 | 79.8 ± 8.9 | 66.4 ± 9.4 | 0.94 ± 0.35 | 66.9 ± 8.0 | 72.2 ± 7.5 |
| LP-tan variant | 1.1 ± 0.35 | 115.5 ± 16.6 | 95.3 ± 11.9 | 0.53 ± 0.18 | 60.2 ± 3.5 | 135.6 ± 4.0 |
| SS-tan   | 3.3 ± 0.38 | 69.8 ± 7.5 | 21.2 ± 1.9 | 0.21 ± 0.09 | 64.7 ± 3.6 | 311.1 ± 23.6 |
| SS-tan G384P | 2.4 ± 0.31 | 70.3 ± 11.3 | 29.3 ± 4.5 | 0.41 ± 0.11 | 85.1 ± 3.3 | 197.6 ± 15.8 |
| SS variant | 3.5 ± 0.51 | 60.7 ± 4.6 | 18.3 ± 1.1 | 0.58 ± 0.28 | 104.6 ± 6.7 | 189.3 ± 8.7 |

Each experiment was performed in quintuplicate, and the averages were used to build this table. In all the cases, the observed wild-type tannase (LP-tan and SS-tan) activity was defined as 100%.
tannic acid (Table 3). The activity assay also showed the similar results (Table 2). Based on these results, we can suggest that the flap in LP-tan can help to bind the small substrates, like methyl gallate; but the substrates with two or more than two aromatic rings, the extra aromatic rings out the activity center may form hydrophobic interactions to prevent the binding.

For SS-tan, the flap-like domain only worked on the binding of tannic acid. So the flap-like domain in SS-tan may have a flexible structure like the esterases, when SS-tan binds large substrates like tannic acid, the flap-like domain might tend to shift away to expose the catalytic site to accommodate the large substrates and the amino acids in the flap-like domain might facilitate the hydrophobic and hydrogen bond interactions with the substrates, which combined with the two hydrogen bond networks to stabilize the binding. While the enzyme binds small substrates like methyl gallate, the flap-like domain might tend to hang above the catalytic center and forge to strengthen the small substrates binding. Such a finding maybe help to guide the application of tannases and provide the theoretical basis for the modification for tannases.

Authors’ contributions
WO, LY and LD performed out the whole study and participated in the design. HX and ZQ participated in the experiments. WM was responsible for initiation and supervision of the study. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The data supporting the conclusions are presented in the main article.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

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