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Expression, purification, crystallization and preliminary X-ray analysis of tannase from *Lactobacillus plantarum*

Tannase catalyses the hydrolysis of the galloyl ester bond of tannins to release gallic acid. It belongs to the serine esterases and has wide applications in the food, feed, beverage, pharmaceutical and chemical industries. The tannase from *Lactobacillus plantarum* was cloned, expressed and purified. The protein was crystallized by the sitting-drop vapour-diffusion method with microseeding. The crystals belonged to space group *P*1, with unit-cell parameters $a = 46.5$, $b = 62.8$, $c = 83.8$ Å, $\alpha = 70.4$, $\beta = 86.0$, $\gamma = 79.4^\circ$. Although the enzyme exists mainly as a monomer in solution, it forms a dimer in the asymmetric unit of the crystal. The crystals diffracted to beyond 1.60 Å resolution using synchrotron radiation and a complete data set was collected to 1.65 Å resolution.

1. Introduction

Tannins are water-soluble polyphenolic compounds that are widely distributed in the plant kingdom and often found in root, wood, bark, fruit and leaves (White, 1957). With molecular masses ranging from 3000 to 20 000 Da, they can be divided into two major groups: the hydrolysable and the condensed tannins. While condensed tannins are nonhydrolysable polymeric flavonoids, hydrolysable tannins are derivatives of gallic acid (3,4,5-trihydroxybenzoic acid) or its *meta*-depsidic forms that are esterified to polyol, catechin or triterpenoid units (Khanbabaee & Ree, 2001). Tannins are best known for their ability to bind and precipitate proteins as exemplified by their long history of usage in tanning (White, 1957). Their ample phenolic rings and phenol hydroxyl groups are thought to form stacking interactions and hydrogen bonds with protein residues (White, 1957; Baxter *et al.*, 1997; Jöbstl *et al.*, 2004). Tannin phenolics are also efficient chelators for metal ions whereby they decrease the biological availability of these indispensable elements (McDonald *et al.*, 1996). As a result, tannins have been considered antinutritional and antimicrobial substances that can function to protect plants against herbivores, pathogens and rots (Haslam, 1988; Scalbert, 1991; Butler, 1992).

Although tannins are generally recalcitrant to biodegradation, various microorganisms have evolved to exploit tannins as carbon and energy sources for growth, using a family of inducible enzymes, the tannin acyl hydrolases, commonly known as tannases (Aguilar *et al.*, 2007). Tannase (EC 3.1.1.20) was first isolated by Fernbach in 1901 from the fungus *Aspergillus niger* (Fernbach, 1901). It belongs to the serine esterases, catalysing the hydrolysis of the ester bond (galloyl ester of an alcohol moiety) and depside bond (galloyl ester of gallic acid) in tannins to release gallic acid (Yamada *et al.*, 1968). Tannase has the potential for wide applications in the food, feed, beverage, pharmaceutical and chemical industries (Lekha & Lonsane, 1997). It is used as a clarifying agent for instant tea, wine, fruit juices, beer and other products to resolve undesirable turbidity caused by the polymerization or complexation with other molecules by tannins (Lekha & Lonsane, 1997; Rodríguez-Durán *et al.*, 2011). Tannase has also been used in the detannification of food and animal feed for nutritional improvement, the homogenization of tannins for high-grade leather making and for the biodegradation of tannin-contaminated waste waters in agriculture and the leather industry (Lekha & Lonsane, 1997; Aguilar & Gutierrez-Sanchez, 2001; Rodríguez-Durán *et al.*, 2011). Another important application of tannase is the hydrolysis of natural tannin-rich materials to produce gallic acid,

which is the intermediate for the manufacture of antibacterial drugs and other widely used gallic acid derivatives (Lekha & Lonsane, 1997; Rodríguez-Durán *et al.*, 2011).

The search for new tannase-producing microbial strains with desired properties, such as altered optimum pH or activity, has been the focus of studies on tannase since its discovery. The selected strains are often employed directly in applications without isolating the enzyme or where only crude enzyme extract was prepared. To date, most of the work on tannase has been on tannases from fungi, especially the *Aspergillus* species, although yeasts and bacteria have gained more attention in recent years (Aguilar *et al.*, 2007). To date, little is known about tannase at the molecular level and only very few tannase genes have been cloned and tested for recombinant expression (Hatamoto *et al.*, 1996; Iwamoto *et al.*, 2008; Böer *et al.*, 2009). The enzyme from the bacterium *Lactobacillus plantarum* is the only one that has been expressed efficiently in recombinant form (Iwamoto *et al.*, 2008). Here, we report for the first time the crystallization and preliminary structure analysis of a tannase from *L. plantarum*, with the aim of determining its three-dimensional structure in order to provide a molecular basis for the understanding and modification of tannase to suit its diverse applications.

2. Materials and methods

2.1. Cloning and expression

The tannase gene (GenBank accession No. AB379685) was amplified by PCR from *L. plantarum* SICC 1.15 strain purchased from the Southwest Center of Industrial Culture Collection in Chengdu, People's Republic of China. The PCR product was inserted into the expression vector pNIC28-Bsa4 (GenBank accession No. EF198106) using the ligation-independent cloning technique (Aslanidis & Jong, 1990). The resulting construct encodes the full-length protein preceded by an extra 22 amino-acid fusion peptide consisting of an N-terminal hexahistidine tag and a tobacco etch virus (TEV) protease cleavable tag.

The expression construct was transformed into *Escherichia coli* BL21-AI cells (Invitrogen, California, USA) and cultured on a 1 × YT agar plate containing 50 µg ml⁻¹ kanamycin. After incubation at 310 K overnight, single colonies were picked and tested for over-expression of the tannase. The selected transformant was cultured in 2 × YT medium at 310 K and protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.7 mM when cell growth reached the exponential phase. After induction the cells grew at 291 K for about 20 h and were harvested by centrifugation.

2.2. Purification and TEV cleavage

The cells were resuspended in lysis buffer consisting of 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM imidazole, 2 mM MgCl₂, 5 mM DTT, 1 mM PMSF (phenylmethanesulfonyl fluoride), EDTA-free protease-inhibitor cocktail tablets (Roche, one tablet per 50 ml lysis buffer), 0.5 mg ml⁻¹ lysozyme, 12 U ml⁻¹ benzonase (Novagen). They were lysed by three passes through an Emulsiflex C5 high-pressure homogenizer (Avestin, Canada) operating at 100 MPa and 277 K. The cell debris was removed by centrifugation (20 000 rev min⁻¹ for 20 min at 277 K). The supernatant was filtered through a 5.0 µm syringe filter and the tagged tannase was purified using immobilized nickel-affinity chromatography followed by gel filtration. Briefly, the clarified bacterial lysate was loaded onto a 5 ml HisTrap column (GE Healthcare) equilibrated with buffer consisting of 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM imidazole, 5 mM

DTT. The column was subsequently washed with the above equilibration buffer containing 20 mM imidazole and eluted with 250 mM imidazole. The collected peak fraction was concentrated and applied onto a HiLoad 16/60 Superdex 200 preparative-grade gel-filtration column (GE Healthcare) pre-equilibrated with 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM DTT. The purified protein was digested with in-house-prepared TEV protease to remove the 22 amino-acid N-terminal tag before crystallization trials. The TEV protease was prepared according to the protocol described by Tropea *et al.* (2009). A clean cleavage of the N-terminal tag was achieved after incubation with TEV protease at 277 K overnight in a buffer consisting of 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA using a TEV protease:protein mass ratio of 1:25. The protein sample was then loaded again onto a HisTrap column to remove the cleaved N-terminal tag and TEV protease that were retained upon passing through the column. The collected protein was concentrated to about 30 mg ml⁻¹ using an Amicon Ultra 10K centrifugal filter (Millipore) in buffer consisting of 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT. The concentrated protein was stored at 193 K in aliquots.

2.3. Crystallization

Crystallization conditions were screened by the sitting-drop vapour-diffusion method at the CSIRO Collaborative Crystallization Centre (C3), Melbourne, Australia using the PACT/JCSG-plus strategy at both 281 and 293 K (Newman *et al.*, 2005). Each droplet had a volume of 300 nl with an equal mixture of the protein and reservoir solution and was set up with a Phoenix robot (Art Robbins Industries). The droplet was equilibrated against 50 µl reservoir solution in 96-well plates (IDEX Corporation). Protein concentrations of 5.5 and 12 mg ml⁻¹ in the original buffer were used in initial screenings based on the results of the Pre-Crystallization Test (Hampton Research). Plate-shaped crystals appeared beginning on day 3 in droplets at 281 K with reservoirs containing 20% (w/v) PEG 3000, PEG 6000 or PEG 8000 as the precipitant and 0.1 M trisodium citrate pH 5.5, citric acid pH 4.0 or phosphate citrate pH 4.2 as the buffer, respectively. The crystals were commonly clustered together and could not be well resolved with optimization of the original conditions by a fine grid search or the addition of organic additives.

Microseeding was employed to obtain single crystals suitable for X-ray analysis. Seeds were produced from the crystals grown in the sitting drop at 281 K that contained a protein concentration of 5.5 mg ml⁻¹ and a reservoir solution consisting of 0.1 M trisodium citrate pH 5.5 and 20% PEG 3000. The crystals from the droplet were placed in 50 µl reservoir solution in an Eppendorf tube with a seed-bead (Hampton Research) and vortexed for 2 min at full speed followed by the addition of another 250 µl reservoir solution. The solution was vortexed briefly and used as the seed stock. Seeding was performed with the dual-dispenser technique using the Phoenix and Mosquito (TTP LabTech) robots and 96-well sitting-drop plates (Newman *et al.*, 2008). Each droplet was set up by mixing 200 nl protein solution at a concentration of 5.5 mg ml⁻¹ in the original buffer, 30 nl seeds and 170 nl reservoir solution consisting of the above components or 14.6% (w/v) PEG 8000 in 0.1 M trisodium citrate pH 5.74. In some reservoir wells, 0.1% dioxane or 4% 1-propanol was included as an additive.

2.4. Data collection and preliminary X-ray analysis

The crystal was flash-cooled directly to 100 K and exposed to X-rays, using a cryoprotectant solution consisting of the original reservoir solution supplemented with 20% (w/v) PEG 200 and then exposed to X-rays. A native diffraction data set was collected on the

Table 1

Diffraction data-collection and processing statistics.

Values in parentheses are for the highest-resolution shell.

Beamline	MX2, Australian Synchrotron
Wavelength (Å)	0.9537
Space group	<i>P</i> 1
Unit-cell parameters (Å, °)	<i>a</i> = 46.5, <i>b</i> = 62.8, <i>c</i> = 83.8, <i>α</i> = 70.4, <i>β</i> = 86.0, <i>γ</i> = 79.4
Resolution (Å)	1.65 (1.74–1.65)
Total observations	391570 (53624)
Unique reflections	100941 (13776)
Multiplicity	3.9 (3.9)
Mean <i>I</i> / <i>σ</i> (<i>I</i>)	12.0 (2.9)
Completeness (%)	95.7 (88.2)
<i>R</i> _{merge} [†] (%)	7.7 (55.0)

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observation of reflection intensity and $\langle I(hkl) \rangle$ is the weighted average intensity for multiple and symmetry-related measurements.

MX2 beamline using an ADSC Quantum 315 detector at the Australian Synchrotron (Melbourne, Australia). The data set consisted of 360 images collected with a 1° oscillation range at a wavelength of 0.9537 Å and a crystal-to-detector distance of 240 mm. The data were processed with the program *XDS* (Kabsch, 2010). The

(a)

(b)

Figure 1

L. plantarum tannase crystals before (a) and after (b) microseeding. The maximum dimension of the largest crystal in (b) is about 0.5 mm. Because the crystals are very thin plates, it was not possible to measure the thickness.

obtained *hkl* intensities were input into the *CCP4* program package for space-group examination, intensity scaling and reduction (Winn *et al.*, 2011). A self-rotation function was calculated with *GLRF* (Tong & Rossmann, 1997) to check the local symmetry in the crystal using data in the resolution range of 10–3.0 Å with an integration radius of 20 Å. A summary of the diffraction data statistics is shown in Table 1.

3. Results and discussion

Recombinant tannase expressed very well in *E. coli*. About 90 mg of protein per litre of medium was purified after the cleavage of the N-terminal tag. While initial screening resulted in clustered crystals, microseeding greatly improved crystallization and yielded crystals suitable for X-ray analysis (Figs. 1a, 1b). Single crystals appeared in droplets within 1 week after seeding and grew to a maximum dimension of about 0.5 mm in 4 weeks. Owing to their plate shape the crystals diffracted anisotropically to a maximum resolution of beyond 1.6 Å using synchrotron radiation. A complete native data set was collected to a resolution of 1.65 Å. Auto-indexing and Laue-group examination revealed that the crystal belonged to space group *P*1, with unit-cell parameters *a* = 46.5, *b* = 62.8, *c* = 83.8 Å, *α* = 70.4, *β* = 86.0, *γ* = 79.4°. The data set has a cumulative merging *R* factor of 0.077 and completeness of 95.7%. Data-collection and processing statistics are shown in Table 1.

After the removal of the N-terminal tag, tannase has a calculated molecular mass of 50 777 Da. Assuming one dimer in the asymmetric unit, the crystal has a solvent content of 44.9%, corresponding to a *V*_M value of 2.23 Å³ Da^{−1}, which is within the most probable range of values observed for proteins (Matthews, 1968). To check the local symmetry in the crystal, a self-rotation function was calculated using the polar angle system (Rossmann & Blow, 1962). A single clear peak was revealed with *κ* = 180° at the position of *φ* = 169° and *ψ* = 115°, verifying that the tannase exists as a dimer in the crystal (Fig. 2). Gel-filtration chromatography analysis of the purified protein revealed

Figure 2

Stereographic plot of the self-rotation function at *κ* = 180° calculated using the program *GLRF* (Tong & Rossmann, 1997) with data between 10 and 3.0 Å. The peak at the position (*φ*, *ψ*) = (169°, 115°) corresponds to the local twofold axis. The map is contoured from 5.0σ in steps of 1.0σ.

Figure 3

Gel-filtration chromatography analysis of *L. plantarum* tannase on a HiLoad 16/60 Superdex 200 column. (a) The UV chromatogram of tannase showing two elution peaks. (b) Calibration curve of the column. The molecular-mass markers are depicted as filled squares and include bovine thyroglobulin (670 kDa), bovine γ -globulin (158 kDa), chicken ovalbumin (44 kDa) and horse myoglobin (17 kDa). The positions corresponding to peaks 1 and 2 are shown as open triangles, indicating the existence of both monomeric and dimeric tannase in solution.

two peaks, the molecular masses of which were calculated as 98.9 and 46.1 kDa from the calibration curve (Figs. 3a, 3b). Fractions from both peaks showed one band at the same position on SDS-PAGE with a molecular weight corresponding to that of monomeric tannase

(data not shown). It was therefore concluded that the tannase exists as both a dimer and a monomer in solution, although the latter is the predominant form as shown in Fig. 3(a). The primary structure of the tannase shows no sequence similarity to any known structures in the PDB. Preparation of heavy-atom derivative crystals for structure determination is under way.

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