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Isolation, Purification, and Some Properties of *Penicillium chrysogenum* Tannase

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Tannase isolated from *Penicillium chrysogenum* was purified 24-fold with 18.5% recovery after ammonium sulfate precipitation, DEAE-cellulose column chromatography, and Sephadex G-200 gel filtration. Optimum enzyme activity was recorded at pH 5.0 to 6.0 and at 30 to 40°C. The enzyme was stable up to 30°C and within the pH range of 4.0 to 6.5. The K_m value was found to be 0.48×10^{-4} M when tannic acid was used as the substrate. Metal salts at 20 mM inhibited the enzyme to different levels.

Fungi hydrolyze tannins during the vegetable tanning process, resulting in considerable economic loss. Tannins are hydrolyzed to gallic acid by the enzyme tannase which is produced by certain fungi. Gallic acid has various industrial and technological applications, and a considerable amount of gallic acid is imported to India. An attempt to produce gallic acid from myrobalan tannin by employing fungal species was unsuccessful, as gallic acid produced during hydrolysis of tannin was found to be decomposed by some other enzyme system in the fungi. Purified tannase was, however, reported to produce gallic acid from tannins (2). Existing literature reveals the isolation, purification, and properties of tannase from *Aspergillus* strains (1, 5, 7, 8, 10, 13), yeasts (2, 3), and plants (12). Although *Penicillium* species grow well in tan liquors and are known to produce tannase, little information is available on the isolation, purification, and properties of tannase obtained from this source.

Penicillium chrysogenum NCIM-722 was collected from the National Collection of Industrial Microorganisms, Poona, India, and was found to grow on different tan liquors, producing a considerable amount of tannase. The organism was preserved in potato glucose agar slants, subcultured every 10th day, and used for the enzyme production. The basal medium used for the enzyme production was Czapek-Dox (11) liquid medium to which tannic acid was added to give a concentration of 2%. The pH of the medium was adjusted to 5.8, and the inoculated culture flasks were incubated at 28°C for 5 days for tannase production.

After the incubation period, the mycelial mat was removed and washed with cold water and homogenized, together with acid-washed sand, in a chilled mortar, using 0.01 M acetate buffer (pH 5.0). The homogenate was centrifuged at 8,000 × *g* for 15 min, and the supernatant was collected and used as the enzyme extract.

Tannase activity was determined by following the method reported by Iibuchi et al. (6). Enzyme solution (0.5 ml) was added to 2 ml of 0.35% (wt/vol) purified tannic acid in 0.05 M citrate buffer (pH 6) taken in a test tube. Then 0.1 ml of the reaction mixture was taken out, and 10 ml of 95% ethanol was added to it to stop the enzyme reaction. The absorbance at 310 nm was read immediately (t_1). After a specific period of enzyme action (t_2) at 30°C, 0.1 ml of the reaction mixture was again taken out, the enzyme reaction was stopped, and the absorbance was read as before at 310 nm. The difference between the absorbancies at t_1 and t_2 was determined. One unit of enzyme activity was determined to be the amount of enzyme required to hydrolyze 1 μmol of the ester bond in 1 min. Enzyme activity values are expressed as units per milliliter unless otherwise specified. Protein was determined by the method of Lowry et al. (9).

The enzyme was purified to homogeneity by ammonium sulfate precipitation, followed by DEAE-cellulose column chromatography and gel filtration on a Sephadex G-200 column, all carried out at 5°C. Solid ammonium sulfate was added to the enzyme extract to 100% saturation; the pH was adjusted to 5.0 and kept there for 30 min; and the mixture was centrifuged at 18,000 × *g* for 30 min. The precipitate was collected, dissolved in 0.01 M acetate buffer (pH 5.0), dialyzed against the same buffer for 48 h, and centrifuged again to remove the insoluble materials. The supernatant was collected and applied to a column (50 by 2.5 cm) of DEAE-cellulose equilibrated with 0.02 M acetate buffer (pH 5.0). The enzyme was eluted with the same buffer containing a linear gradient of NaCl. Active

TABLE 1. Summary of purification of *P. chrysogenum* tannase

Fraction	Vol (ml)	Total protein (mg)	Total activity (U × 10^3)	Sp act (U/mg)	Purification (fold)	Yield (%)
Mycelial extract	800	2,981.6	10.8	3.6	1.0	100.00
Ammonium sulfate precipitation	125	810.0	7.5	9.2	2.5	69.0
DEAE-cellulose column chromatography	90	120.0	3.3	27.0	7.5	30.0
Sephadex G-200 gel filtration	35	23.0	2.0	86.0	24.0	18.5

fractions were collected, dialyzed against 0.01 M acetate buffer (pH 5.0), and applied to a column (50 by 2.0 cm) of Sephadex G-200 equilibrated with 0.02 M acetate buffer (pH 5.0) containing 0.5 M sodium chloride. The enzyme was eluted with the equilibrating buffer of the column, and 5-ml fractions were collected at a flow rate of 20 ml/h. Active fractions were dialyzed against 0.01 M acetate buffer (pH 5.0), lyophilized, and stored at 4°C for further studies.

The enzyme was found to be homogeneous on polyacrylamide (4) and sodium dodecyl sulfate-polyacrylamide (12) gel electrophoresis. After the final purification, a 24-fold purity of the enzyme was achieved with 18.5% enzyme recovery (Table 1).

The purified enzyme showed a broad pH dependence, with optimum activity at pH 5.0 to 6.0, and the enzyme was stable in the pH range of 4 to 6.5 at 16°C. The optimum pHs for tannase activity were reported to be 5.0 to 5.5, 5.5, and 3.5 to 7.5 in cases of enzymes isolated from *Aspergillus flavus* (1), *Aspergillus oryzae* (7), and the yeast *Candida* sp. strain K-1 (2), respectively. Tannases from *A. flavus* and *A. oryzae* were found to be stable at pH ranges of 5.0 to 5.5 at 30°C and 4.5 to 6.0 at 5°C, respectively.

The optimum temperature for the enzyme activity was found to be 30 to 40°C. The enzyme was stable up to 30°C and 95% stability was observed up to 45°C. Similar observations were reported for tannase from *A. oryzae* (7). The K_m value of *P. chrysogenum* tannase for tannic acid was observed to be 0.48×10^{-4} M. A K_m value of 0.5×10^{-4} M was reported by Yamada et al. (13) with purified tannase isolated from *A. flavus*.

TABLE 2. Effect of metal salts on tannase activity

Metal salt used (20 mM concn)	% Inhibition of enzyme activity
Control (no metal salts)	0
$ZnCl_2$	22
$ZnSO_4 \cdot 7H_2O$	45
$FeSO_4 \cdot 7H_2O$	45
$MgSO_4 \cdot 7H_2O$	17
$MnSO_4 \cdot 4H_2O$	22
$CuSO_4 \cdot 5H_2O$	53
$NiCl_2 \cdot 6H_2O$	42
$Co(NO_3) \cdot 6H_2O$	36
$Ca(CH_3COO)_2$	33

Tannase activity was inhibited to a maximum of 53% by Cu^{2+} followed by Zn^{2+} and Fe^{2+} and to a minimum of 17% by Mg^{2+} at 20 nM (Table 2). Similar inhibitory action by mineral salts was also noted in the case of *A. oryzae* (7) tannase.

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