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Tannin acyl hydrolase (EC 3.1.1.20) activity of *Aspergillus, Penicillium, Fusarium* and *Trichoderma*

B. Bajpai and S. Patil*

A spectrophotometric method to determine gallic acid, residual gallotannin and tannin acyl hydrolase (EC 3.1.1.20) activity during microbial hydrolysis of pentagalloyl glucose is described. The following equations have been developed to estimate gallotannin and gallic acid in the incubation medium by absorbance measurements at two different wavelengths: concentration of gallotannin ($\mu g\ ml^{-1}$) = 34.41 ($A_{293.8}$) − 6.98 ($A_{254.6}$); concentration of gallic acid ($\mu g\ ml^{-1}$) = 21.77 ($A_{254.6}$) − 17.17 ($A_{293.8}$). As compared to *Aspergillus* and *Penicillium*, the fungal genera extensively studied for the production of this enzyme, *Fusarium solanii* and *Trichoderma viride* exhibited higher enzyme activity showing approximately 88 and 84 mole percent conversion respectively after a 24 h incubation period.

Key words: Gallic acid, gallotannin, tannase, tannin acyl hydrolase.

Tannin acyl hydrolase (EC 3.1.1.20), commonly referred as tannase, is an inducible enzyme produced by fungi, mainly *Aspergillus* and *Penicillium* species (Iibuchi *et al.* 1967; Rajkumar & Nandy 1983; Kawakubo *et al.* 1991; Lekha & Lonsane 1994), bacteria (Deschamps *et al.* 1983) and yeast (Aoki *et al.* 1976). The major commercial application of this enzyme is in the hydrolysis of gallotannin to gallic acid, a key intermediate required for the synthesis of an antifolic antibacterial drug, trimethoprim (Sittig 1988). Tannase is also used as a clarifying agent in the wine, fruit juices and coffee-flavoured soft drink industries (Lekha *et al.* 1993).

Few spectrophotometric methods are available for the estimation of tannase activity of microorganisms. Methods based on artificial substrates such as methyl gallate (Dhar & Bose 1964) and *p*-nitrophenyl gallic acid (Haslam & Tanner 1970) do not represent the actual gallotannin-hydrolysing capacity of microorganisms. Changes in absorbance at 310 nm (Iibuchi *et al.* 1967) and absorption at 260 nm after the protein precipitation of residual gallotannin (Deschamps *et al.* 1983) have been used, but gallic acid formed as a result of gallotannin hydrolysis undergoes further breakdown through the tricarboxylic acid cycle in *A. niger*

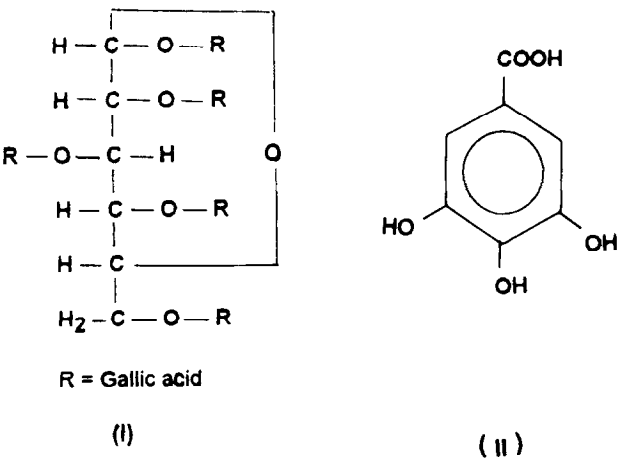

Figure 1. Structures of pentagalloyl glucose (I) and gallic acid (II).

(Watanabe 1965). Therefore, none of the available methods is applicable for the estimation of actual tannase activity and determination of residual gallotannin and gallic acid formed as a result of enzymic hydrolysis. The present communication reports a method for the simultaneous estimation of residual gallotannin and gallic acid formed and subsequent determination of tannase activity. Further, it compares the tannase activity of *Aspergillus* and *Penicillium*

The authors are with the School of Life Sciences, Devi Ahilya University, Vigyan Bhawan, Khandwa Road Campus, Indore-452 001, India.* Corresponding author.

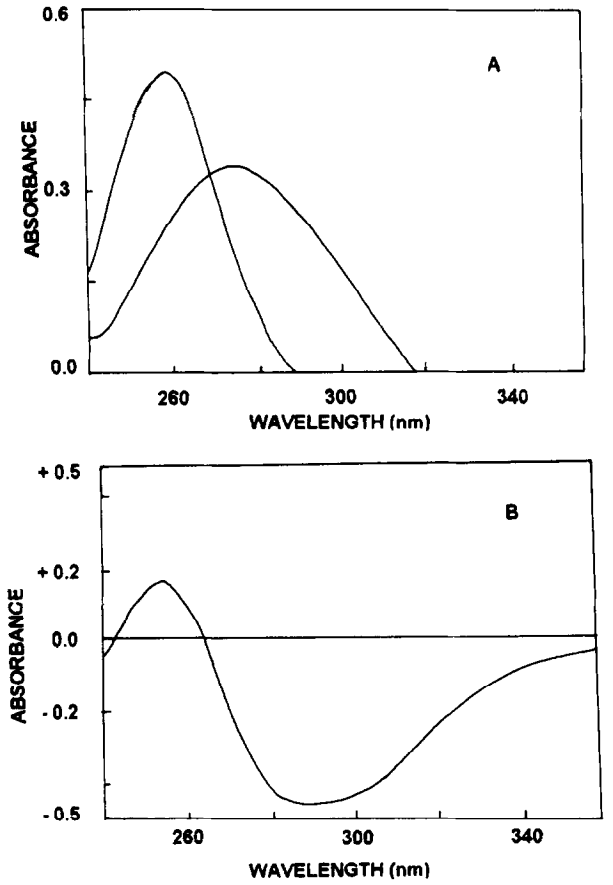

Figure 2. Overlay spectra of 10 μg ml^{-1} gallotannin and gallic acid (A) and difference spectrum between gallotannin and gallic acid (B) in 0.5 M acetate buffer, pH 6.0.

with *Fusarium* and *Trichoderma*, two fungal genera so far not reported to produce this enzyme.

Materials and Methods

The overlay and difference spectra of gallotannin (pentagalloyl glucose from *Quercus infectoria*, supplied by Kisalaya Pharmaceuticals, Pithampur, India) and gallic acid (Sigma, USA) were recorded in 0.5 M acetate buffer, pH 6.0, on a Shimadzu 160-A double-beam spectrophotometer. The specific extinction coefficients of gallotannin and gallic acid were determined at 254.6 nm and 293.8 nm and equations were developed by the two wavelength calculation method to estimate the amount of gallotannin and gallic acid present in a standard binary mixture.

Organisms

Fungi used in the present study were obtained from the Microbial Type Culture Collection, Chandigarh, India and maintained on potato dextrose agar slants supplemented with 0.02% gallotannin. The medium used for growing fungi contained (g/l): sucrose, 30.0; NH_4NO_3, 1.65; KNO_3, 1.9; $MgSO_4.7H_2O$, 0.37; $CaCl_2.2H_2O$, 0.44; KH_2PO_4, 0.17 and (mg/l): H_3BO_3, 6.2; $MnSO_4.H_2O$, 16.9; $ZnSO_4.7H_2O$, 8.6; $Na_2MoO_4.2H_2O$, 0.25; $CuSO_4.5H_2O$, 0.025; $CoCl_2.6H_2O$, 0.025; $FeSO_4.7H_2O$, 5.5; and Na_2EDTA, 7.6. The medium was supplemented with 5 g gallotannin/litre and adjusted to pH 5.6 with 1 N HCl. The fungi were grown in 250 ml Erlenmeyer flasks containing 50 ml sterile medium on a rotatory shaker (160 rev. min^{-1}) at 30 ± 2°C.

Estimation of Tannase activity

Samples of the incubation medium were filtered through Whatman No. 1 paper and the filtrate (0.1 ml) was diluted 100 fold with 0.5 M acetate buffer, pH 6.0 and absorbance was recorded at preselected wavelengths. The concentrations of gallotannin and gallic acid were then calculated. One unit of enzyme activity is defined as the amount of enzyme liberating 1 μg gallic acid ml^{-1}/min.

Recovery and identification

The fungi were grown as described above in 500 ml flasks containing 100 ml medium. After 48 h, 5 g gallotannin, dissolved in 5 ml sterile distilled water, was added to each flask. The incubation was terminated after a further 48 h and the medium was filtered, adjusted to pH 2.7 with 1 N HCl and chilled to −5°C. The precipitate which formed after thawing the frozen medium was vacuum filtered, dissolved in warm distilled water and decolourized with charcoal. The crystalline needles separated on cooling, were collected and then subjected to qualitative analysis.

Results and Discussion

The overlay spectra and difference spectra of gallotannin and gallic acid in acetate buffer are given in Figures 2A and 2B respectively. The wavelengths of maximum difference in the absorbance of these compounds were 254.6 nm and 293.8 nm. The specific extinction coefficients at these wavelengths were found to be 273 and 346 for gallotannin and 547 and 111 for gallic acid respectively. Using the specific extinction coefficients, the following equations were developed for the estimation of gallotannin and gallic acid:

$$\text{Concentration of gallotannin } (\mu g\ ml^{-1}) = 34.41\ (A_{293.8}) - 6.98\ (A_{254.6}) \quad (1)$$

$$\text{Concentration of gallic acid } (\mu g\ ml^{-1}) = 21.77\ (A_{254.6}) - 17.17\ (A_{293.8}) \quad (2)$$

where A indicates absorbance at respective wavelengths.

To test the feasibility of the above equations, the concentrations of gallic acid and gallotannin present in different proportions in standard binary mixtures were determined and are presented in Table 1. The percentage error recorded in actual and estimated amounts of gallotannin and gallic acid present in the standard binary mixtures ranged from +0.6 to −3.05, which appears to be within the limits of experimental error. However, the method cannot be applied for the estimation in case of fungus producing secondary metabolites absorbing at these wavelengths. The absence of such metabolites in the fungi included in the present study has been confirmed by including appropriate blanks without gallotannin in the incubation medium.

The tannase activity of some fungi calculated on the basis of gallic acid formed after 24 h and 48 h incubation periods is shown in Table 2. It is evident from the data in

Table 1. Actual and estimated amounts of gallotannin and gallic acid present in various proportions in standard binary mixtures.

Actual amounts present ($\mu g\ ml^{-1}$)		Absorbance		Estimated amounts* ($\mu g\ ml^{-1}$) [% Error]	
Gallotannin	Gallic acid	$A_{254.6}$	$A_{293.8}$	Gallotannin	Gallic acid
40.0	0.0	1.085	1.327	39.64 (−0.90)	0.06 (–)
30.0	10.0	1.362	1.145	29.89 (−0.37)	9.99 (−0.10)
20.0	20.0	1.629	0.908	19.87 (−0.65)	19.87 (−0.65)
10.0	30.0	1.891	0.676	10.06 (+0.60)	29.56 (−1.47)
0.0	40.0	2.127	0.438	0.23 (–)	38.78 (−3.05)

*Estimated by equations 1 & 2.

Table 2. Amounts of gallic acid formed, residual gallotannin and tannase activity of fungi after 24 h and 48 h incubation periods (initial gallotannin concentration 5 gl^{-1}).

Organisms	24 h			48 h		
	Gallic acid ($\mu g\ ml^{-1}$)	Residual gallotannin ($\mu g\ ml^{-1}$)	Tannase activity ($U\ ml^{-1}$)	Gallic acid ($\mu g\ ml^{-1}$)	Residual gallotannin ($\mu g\ ml^{-1}$)	Tannase activity ($U\ ml^{-1}$)
A. niger MTCC 282	2293 ± 33	2335 ± 87	1.59	1750 + 31	1395 ± 44	0.61
A. niger MTCC 404	2357 ± 10	1989 ± 15	1.64	2284 ± 26	1901 ± 30	0.79
A. aureus MTCC 151	2372 ± 27	2036 ± 15	1.65	2333 ± 13	1830 ± 41	0.81
A. oryzae MTCC 152	2859 ± 35	1557 ± 27	1.99	3135 ± 27	860 ± 11	1.09
A. fischerii MTCC 150	1455 ± 47	3478 ± 96	1.00	3093 ± 71	1524 ± 48	1.07
A. parasiticus MTCC 411	3264 ± 67	1110 ± 74	2.26	3032 + 82	507 ± 48	1.05
A. terreus MTCC 279	2958 ± 42	1478 ± 34	2.05	2897 ± 44	1251 ± 11	1.01
P. chrysogenum MTCC 161	3352 ± 24	687 ± 48	2.33	2377 ± 72	624 ± 13	0.83
F. solanii MTCC 350	3665 ± 59	545 ± 24	2.55	3808 ± 46	323 ± 46	1.32
T. viride MTCC 167	3521 ± 44	700 ± 66	2.45	3254 ± 16	520 ± 58	1.13

The data represent the mean of three replicates ± standard deviation.

Table 2 that *Fusarium solanii* and *Trichoderma viride* exhibited higher tannase activity as compared to the thoroughly investigated *Aspergillus* and *Penicillium* species. Gallic acid accumulated in the medium showed a further decline after 24 h incubation in the case of *P. chrysogenum* MTCC 161 and all *Aspergillus* species studied, with the exception of *A. oryzae* MTCC 152 and *A. fischerii* MTCC 150, indicating its further breakdown. *Fusarium solanii* MTCC 350 and *Trichoderma viride* MTCC 167 showed the highest tannase activity; about 88 and 84 mole percent conversion was obtained after 24 h incubation respectively.

The crystalline product recovered from gallotannin hydrolysis by all organisms used in the present study showed m.p. 248-253°C, λ_{max}^{EtOH} 269 nm, R_f value 0.69 ± 0.02 on co-TLC with authentic gallic acid on silica gel coated plates developed in ethyl acetate/chloroform/formic acid (4:4:1 v/v). The product of gallotannin hydrolysis was identified as gallic acid.

As compared to extensively studied *Aspergillus* and *Penicillium* species, *Fusarium* and *Trichoderma* showed higher tannase activity. Both these genera should be further investigated for the production of tannase and gallic acid from gallotannin on a commercial scale.

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