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Studies on the oxidation of tannins by *Aspergillus flavus*

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Abstract. A method for the estimation of tannin in presence of catechin, pyrogallol, protocatechuic acid and gallic acid using polyamide column chromatography was developed. Tannin added to the growing culture of *Aspergillus flavus* was oxidised to different extents depending on the duration of incubation. The oxidised compound was identified in the culture filtrate as a polymerised product of tannin.

Keywords. Tannins; nontannins; tannin precipitation; polyamide adsorption; polymerisation; gel filtration.

Introduction

A large number of methods for the determination of tannins and related polyphenols have appeared in the literature. However, none is accurate enough to determine the tannin in presence of non-tannins at a very low concentration. The volumetric permanganate method of Lowenthal (1877) and the colorimetric method of Rosenblatt Peluso (1941) and Pro (1952) have the limitation that the total phenolic hydroxyl groups present in both tannin and non-tannin are estimated. Routinely, hide powder is used to remove the major part of the simple phenols and the bulk of the other major complex materials. In the present study, polyamide has been tried for the adsorption of tannins in presence of nontannins and found to be much more efficient than hide powder.

Natural plant tannins represent a group of high molecular weight polyphenolic materials which are widely distributed throughout the plant kingdom (Hathway, 1962; Turd, 1962). They are generally considered to be resistant to microbial decomposition or polymerisation. Condensed tannins, however are more resistant than hydrolysable tannins (Lewis, 1966; Benoit, 1966). Filamentous fungi, especially, species of the genera *Penicillium* and *Aspergillus* have been implicated in the decomposition of tannin (Nishira and Mugibayashi, 1953, 1956). The production of keto acids (Beveridge and Hugo, 1964; Dykerhoff and Armbruster, 1933; Freudenberg and Vollbrecht, 1921) and of oxalic acid (Hathway, 1962) by degradation of phenolic compounds during the decomposition of tannins were observed. In contrast, Hathway (1958) and Hathway and Seakins (1957) identified the oxidative polymers of the accompanying catechins in which C–C links through the 2 phenyl group appeared to be the main mode of condensation.

The condensed tannins which are used as tanning materials in the leather industry, are normally polymerised by molds and are also decomposed by soil microorganisms. It was therefore of interest to investigate the action of *Aspergillus flavus,*

isolated from soils contaminated with tannery wastes, on condensed tannins and develop a method for estimating tannings.

Materials and methods

Materials

Phloroglucinol, gallic acid, polyamide were purchased from E. Merck, Darmstadt, Germany. Protocatechuic acid and cinchonine sulphate were obtained from Koch-Light Laboratories Ltd., Colnbrook, England and from B.D.H. Chemicals Ltd., Poole, England, respectively. Catechin and rutin were purchased from Zyma, S.A., Nyon, Switzerland. Tannic acid was obtained from Fluka Ag Buchs SO, Switzerland. Caffeine was isolated fro coffee seeds following the method of Sondheimer *et al.* (1961). All other chemicals were of reagent grade.

Methods

Preparation of pure tannin from wattle extract: To a 5% solution of spray-dried wattle extract, a saturated solution of caffeine was added untill the precipitation was complete. The solution was centrifuged, the precipitate was collected and dried under vacuum. The dry material was dissolved in methanol and precipitated with 5 volumes of chloroform. The precipitate was collected under nitrogen. The process was repeated twice. The purity of the tannin powder was tested by thin layer chromatography using butanol; acetic acid; water (4:1:5) solvent system and by spraying with diazotised p-nitroaniline reagent.

Estimation of phenolic compounds and tannin: Standard curves for tannin and other phenolic compounds were constructed using the method of Folin and Ciocalteu (1927). One ml solution of tannin, gallic acid, catechin, protocatechuic acid and phloroglucinol (concentration indicated in figure) in the medium (described under growth condition) 7.0 ml of distilled water was added and the contents were mixed with 1.5 ml of 20% sodium carbonate and 0.5 ml of folin-phenol reagent. The mixture was shaken well, kept at room temperature for 1 h and absorbance was measured at 725 nm in a SP Unicam 1800 spectrophotometer.

Estimation of tannin-phenolics by precipitation: Tannins (0.1-2.5 mg/ml) were precipitated by adding either cinchonine sulphate, caffeine and or lead acetate solutions (Peri and Pompi, 1971); Sunthankar and Jatkar, 1938). The concentrations of the reagents required for completely prescipitating the tannins were determined earlier. The precipitate containing tanin phenolics was dissolved in a known volume of 10% methanol and total phenolic OH groups estimated (Folin and Cicocalteu, 1927).

Estimation of tannin by polyamide adsorption method: Tannin solution (2.5 mg) was adsorbed on to a column of polyamide (1.5×4 cm) equilibrated with 90% methanol. The adsorbed material was eluted successively with 90, 60, 30 and 10% methanol (at a flow rate of 50 ml/h). The total phenolic OH groups were estimated in the eluate as described above.

Organism and growth conditions: The mold *A. flavus* was isolated from the soil collected from tannery wastes disposal area (Mallika *et al.* 1980). The growth medium containing 1 g KH_2 PO_4, 0.5 g $MgSO_4$, 0.2 g KNO_3, 0.2 mg $FeSO_4$ $7H_2O$.

0.2 mg $ZnCl_2$, 0.2 mg $CuSO_4$, $5H_2O$, 0.1 mg $MnSO_4$, $4H_2O$, 10 g glucose, 40 g yeast extract in 1 L water (Mallika *et al.,* 1980), was used for growth of A *flavus* and oxidation of tannin. Tannin (0.1%) was added to the medium separately under asceptic conditions.

The medium was inoculated with a seven day old spore suspension of *A. flavus* in sterile water and the flasks were incubated at room temperature (28-32°C) on a rotary shaker. Controls were carried out side by side in an identical manner but without the mold *A. flavus.*

After 24 h of incubation the media were separated by filtration and the phenolic compounds were estimated before and after polyamide treatment as described above.

Determination of molecular weight by gel filtration: The approximate molecular weight of the tannins and the polymerised product in the culture medium was determined by the method of Gelotte (1960) using sephadex G-25 column, catechin (M_r 290), rutin (M_r 160) and tannic acid (M_r 1701) were used as standards. The aromatic compounds were monitored by measuring the absorbance at 280 nm. From the plot of logarithm of molecular weight versus elution volume, the molecular weights of tannin and polymerized product formed in the culture filtrate of mold *A. flavus* were determined (figure 1).

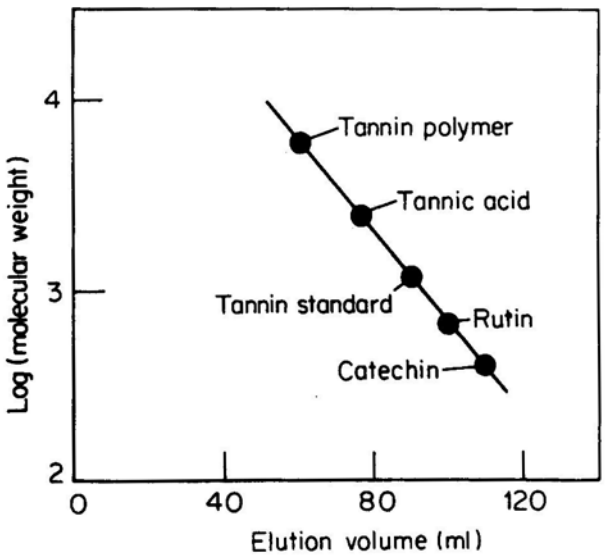

Figure 1. Molecular weight of tannin polymer by gel filtration on Sephadex G-25.
Samples of 5 mg in 5 ml water or 5 ml culture filtrate of *a. flavus* were subjected to Sephadex G-25 gel filtration chromatography. 5 ml fractions were collected at a flow rate of 15 ml/h.

Results and discussion

Oxidation of tannin by A. flavus

As mentioned earlier, tannin (0.1%) was added to the growing culture of *A flavus,* and the amount of tannin oxidised in the medium at various incubation periods by

A. flavus was estimated. The extent of oxidation of tannin was calculated by substract ing the value of total phenolic OH groups in the test solution from that of control (without the organism). The results are shown in table 3.

It is known that vegetable tannin can be precipitated by many chemical reagents and these precipitation techniques have become the tool for the estimation of tannin in any of the vegetable tan liquors or in tannery effluents. It can be seen from table 1 that recovery of tannin to nearly 60% could be achieved by precipitation

Table 1. Estimation of tannin by precipitation methods.

Tannin used (mg/ml)	Percentage recovery of tannin		
	Cinchonine sulphate (0.02%)	Caffeine (0.12%)	Lead acetate (0.06%)
0.10	60.0	40.0	58.0
0.25	50.0	40.0	50.0
0.50	45.5	25.0	44.0
1.00	33.3	22.5	32.0
1.50	32.0	20.0	32.0
2.00	32.0	17.5	27.2
2.50	32.0	8.0	22.0

with both cinchonine sulphate and lead acetate and to about 40% by the addition of caffeins. Recovery of tannin by these precipitation methods decreases appreciably when higher concentrations of tannins are present. Hence, these methods cannot be used for the estimation of tannins, although Peri and Pompi (1971) reported a method of estimating the total phenolics after precipitating simple phenolics, non-tanning flavans, condensed and hydrolysable tannins by precipitating with cinchonine sulphate. In the case of lead acetate precipitation, the separation of precipitated lead salts from other materials was very difficult. Hence, lead acetate precipitation method also cannot be adopted for the estimation of tannins. Sunthankar and Jatkar (1938) reported that precipitation with lead acetate did not separate tannins from non-tannins of myrobalan extracts.

When polyamide was used for the adsorption of tannins, it was observed that 0.5 *g* of polyamide could absorb about 2.5 mg tannins. However, water and 10% methanol eluted only a very insignificant amount of tannin from the column (table 2). Even

Table 2. Sepcificity of polyamide for adsorption of tannin.

Methanol in water for elution (% v/v)	Retention of adsorbed tannin (%)
0	98.90
10	98.90
30	97.30
60	96.10
90	95.00

when 90% methanol in water was used for elution about 95% of tannin was retained on the column. Thus, polyamide column is ideally suited for the estimation of tannins than any of the precipitation methods where the recovery of tannin was only 32% in cinchonine sulphate, 8% in caffeine and 22% in lead acetate precipitation.

When a mixture of tannins and nontannin phenolics was passed through the polyamide column, almost 99% of all non-tannin phenolics were not retained in the column whereas the tannin was adsorbed by polyamide. The removal of polyphenolic substances like flavonols by the use of polyacaprolactum powder has been demonstrated by Sanderson (1964).

Table 3. Oxidation of tannin by *A. flavus*.

Period of oxidation (h)	Tannin oxidised (%)
24	52.9
48	85.3
72	85.3
96	85.3

Having established the reliability of the polyamide adsorption method, it was used to study the oxidation of tannin by the *A. flavus.* After incubting the tannin extract with *A. flavus* and the oxidised product was passed through the polyamide column, all of it was retained on the column. It can be seen from table 3 that about 53% tannin was oxidised by *A. flavus* in 24 h. After a period of 48 h, the oxidation of tannin was maximum (85%). Addition incubation period had no effect on oxidation of the tannin.

The fact that all the reaction product was adsorbed on to the polyamide column indicated that tannin was not degraded to a simpler phenolic compound by *A. flavus,* but instead, a higher mol. wt. compound may be have been formed. The determination of the molecular weight of the product by Sephadex G-25 gel filtration, only two compounds, one polymerised product corresponding, to M_r of 4600 and the other unreacted tannin corresponding to a M_r of 1200 (figure 1) which is the mol. wt. of pure tannin from wattle extract were obtained. Polymerisation of tannins reduces the number of phenolig hydrosyl groups due to the formation of quinone (Swam and Hills, 1959; Smit *et al.,* 1955; Kursenov and Zeprometov, 1949).
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Polymerisation of tannin by the mold *A . flavus* may have occured through quinine formation, as indicated by the maximum absorption of the polymerised product at 420 nm. Thus it is evident from these studies that *A. flavus* could polymerise tannin to a compound of higher molecular weight.

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