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SPECTROPHOTOMETRIC ASSAY OF TANNASE

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Abstract—The synthesis of the *o*, *m* and *p*-nitrophenyl esters of gallic acid and 3-*O*-methylgallic acid is described. The use of the *p*-nitrophenyl esters as substrates in a spectrophotometric assay of the enzyme tannase is described.

INTRODUCTION

TANNASE is an inducible enzyme formed in the mycelia of moulds of the genus *Aspergillus* and *Penicillium* when grown on tannic acid.[1] The enzyme, which is dominant during the early stages of growth, hydrolyses esters of phenolic acids and exhibits clearly defined specificity towards the phenolic acid.[2] Thus it hydrolyses gallate, protocatechuate and 3,5-dihydroxybenzoate esters but not those of salicylic, 2,4-dihydroxybenzoic and 2,5-dihydroxybenzoic acids.[2,3] The enzyme has been widely used as a sensitive analytical probe in structural studies of naturally occurring esters of gallic acid[4] and it may also be functional in the microbial breakdown of such substrates during the early stages of the natural decay of organic matter.[5] Some properties of the enzyme have been reported[3,6-9] but detailed physicochemical studies and surveys of the enzyme's distribution are dependent on a reliable method of assay of activity. Previously this has been achieved by automatic titration of the acid liberated[6] or spectrophotometrically.[10,11] The deficiencies of the former method are well known[12] and the spectroscopic methods,[10,11] based on the small differences in u.v. absorption of gallic acid and its methyl ester or tannic acid, were subject in our hands to considerable error.[13]

RESULTS AND DISCUSSION

Esters of gallic and 3-*O*-methylgallic acid with *o*, *m* and *p*-nitrophenol were prepared by condensation of the appropriate phenol with 3-acetoxy-4,5-diphenylmethylenedioxybenzoyl chloride[14] or 3,4-diphenylmethylenedioxy-5-*O*-methylbenzoyl chloride, followed by treatment with acetic acid to remove the protecting groups. The esters were characterized by i.r.,

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NMR and mass spectroscopy and were all hydrolysed by tannase preparations to the constituent acid and nitrophenol. The *p*-nitrophenyl esters proved to be eminently satisfactory from the point of view of kinetic assay. The reactions were conducted in 20% ethanol solution; below pH 6·2 the change in concentration of free *p*-nitrophenol and above pH 6·2 the change in concentration of its anion were measured at 350 nm and 404 nm respectively and used as a measure of the extent of reaction. In Fig. 1 the u.v. absorption characteristics of *p*-nitrophenylgallate, *p*-nitrophenol and gallic acid in 0·1 M buffer–20% ethanol are shown and these illustrate that assay of tannase activity at pH 7·1, although this is removed from the pH optimum of the enzyme,[2,3] is most accurate using this spectroscopic technique. At this pH a small correction for the spontaneous hydrolysis of the ester was necessary.

Using both *p*-nitrophenyl esters and a standard tannase preparation the variation of the initial rates of the hydrolytic reaction with substrate concentration at pH values from 3·5

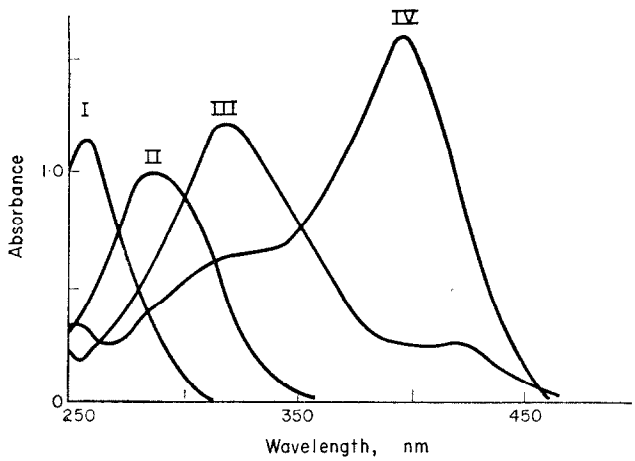

FIG. 1. U.V. AND VISIBLE ABSORPTION SPECTRA IN 20% ETHANOL–0·1 M BUFFER.
I, Gallic acid, $1{\cdot}38 \times 10^{-4}$ M, acetate buffer, pH 6·3.
II, *p*-Nitrophenyl gallate, $5{\cdot}03 \times 10^{-5}$ M, acetate buffer, pH 6·2.
III, *p*-Nitrophenol, $1{\cdot}45 \times 10^{-4}$ M, acetate buffer, pH 6·2.
IV, *p*-Nitrophenol, $1{\cdot}45 \times 10^{-4}$ M, phosphate buffer, pH 7·4.

to 7·5 were measured. Figure 2 shows a plot of the data obtained at pH 7·1 in 0·1 M phosphate buffer. Values of K_m and V_{max}[15] were then obtained by use of a reciprocal plot from Eadie's equation[16]. V_{max} was comparable for both substrates but K_m—the substrate concentration to attain $V_{max}/2$—was approximately twice as great for the ester of 3-*O*-methylgallic acid as for the ester of the parent acid. This may be a reflection of the greater steric bulk of a methyl group as opposed to a hydrogen atom on the substrate molecule leading to a decrease in the stability of the enzyme–substrate complex.

The broad pH optimum of tannase activity (pH 4–5) previously observed[2,3] was confirmed and detailed measurements showed that this is probably composed of two optima (pH 4·2 and pH 4·8), Fig. 3. Since a crude extract of tannase was used for these experiments the two maxima may correspond to the pH optima of different galloyl esterases. In earlier work Rogers[3] observed that purification of tannase by ion-exchange chromatography led

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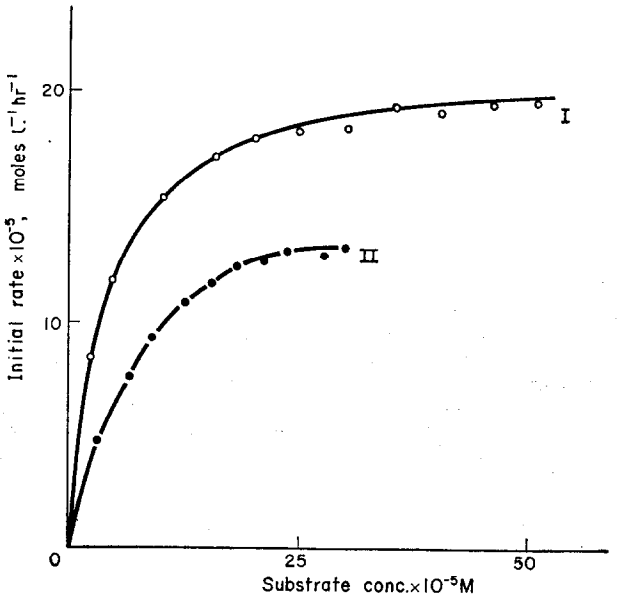

FIG. 2. VARIATION OF INITIAL RATE OF REACTION WITH SUBSTRATE CONCENTRATION, I-*p*-NITROPHENYLGALLATE AND II-*p*-NITROPHENYL-3-*O*-METHYLGALLATE. TANNASE CONCENTRATION 4 μg/ml. Rate of reaction recorded at 404 nm in 20% ethanol–0·1 M phosphate buffer, pH 7·1, at 30°. V_{max} for *p*-nitrophenylgallate, $21{\cdot}1 \times 10^{-5}$ mole $l.^{-1}$ hr^{-1} and K_m, $3{\cdot}91 \times 10^{-5}$ mole $l.^{-1}$. V_{max} for *p*-nitrophenyl-3-*O*-methylgallate, $16{\cdot}9 \times 10^{-5}$ mole $l.^{-1}$ hr^{-1} and K_m, $8{\cdot}2 \times 10^{-5}$ mole $l.^{-1}$.

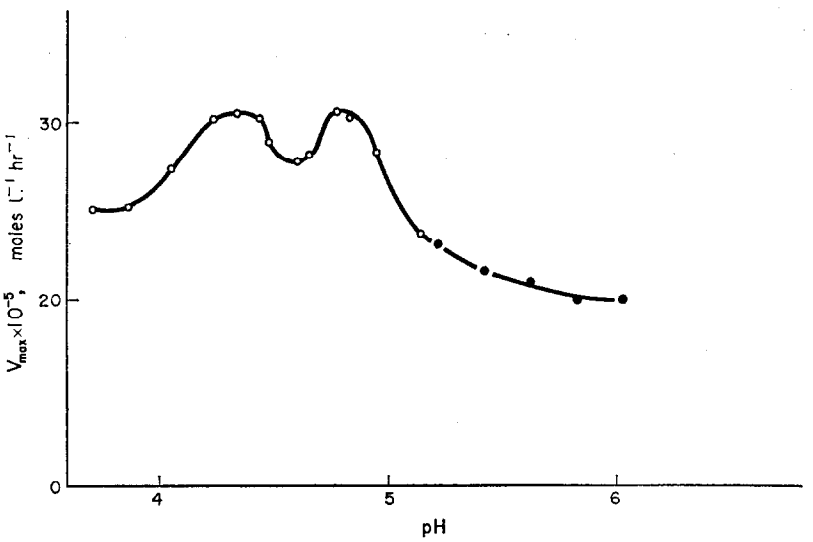

FIG. 3. VARIATION OF TANNASE ACTIVITY WITH pH. *p*-Nitrophenylgallate, $4{\cdot}5 \times 10^{-4}$ M in 20% ethanol–0·1 M buffer (○, acetate; ●, phosphate). Tannase concentration 4 μg/ml.

to the enzyme being eluted in four different fractions each with comparable specificities. Rogers[3] tentatively suggested that the differences between the esterases may result from differences in their pH optima and resulted in the broad pH optimum of the crude enzyme when isolated.

EXPERIMENTAL

Preparation of tannase. The enzyme was prepared using a modified growth medium described by Knudson. Two solutions, I (800 ml; containing $MgSO_4$, $5H_2O$, 0·5 g, KH_2PO_4, 0·1 g, KCl, 0·5 g, and $NaNO_3$, 2·0 g) and II (200 ml; containing B.D.H. tannic acid, 20 g, and $(NH_4)_2SO_4$, 1·0 g), were sterilized, the former by autoclaving and the latter by filtration through Carlson Steripads (iron free, grade E.K.O.). The media (I, 160 ml; II, 40 ml) were transferred aseptically to penicillin flasks and inoculated with *Aspergillus niger* 106 and cultured at 30° in the dark until a uniform black mat of sporangia covered the surface (72–96 hr). The combined mycelia (20–25 g) were washed with distilled water and blended in distilled water (220 ml) at 15° and the resultant suspension shaken for 24 hr at 15°. After the solution had been filtered through acid-washed Kieselguhr it was dialysed for 20 hr at 2° against M/1000 acetate buffer, pH 5·0, and then freeze dried. The crude tannase (0·48 g) was dissolved in water (50 ml) and the light-brown solution stored at −20°. The total protein content, determined by the method of Lowry, was 0·25 g.

Assay of tannase activity. Stock solutions of the substrates *p*-nitrophenylgallate and *p*-nitrophenyl-3-*O*-methylgallate were prepared in spectroscopic ethanol and stored at 0°. Acetate and citrate buffer[17] solutions (0·1 M) were used in the range pH 3·5–5·2 and phosphate buffer[17] (0·1 M) in the region pH 5·2–7·5. Spectroscopic measurements were made using a Cary 14 u.v. spectrophotometer containing a thermostatted cell compartment at 30° and the pH of each reaction sample was measured in a Radiometer (PHM 4C) pH meter. Reaction samples (2·5 ml, containing 20% ethanol and 80% buffer solution) with a substrate concentration of 2×10^{-5} M–50×10^{-5} M were prepared immediately prior to use and equilibrated for 5 min in the cell compartment. Tannase solution (2·5–10·0 μl) was added and measurement of u.v. absorption at 404 or 350 nm commenced 15 sec after the addition of the enzyme. Reactions were run for 5 min and the initial rate of reaction obtained graphically. Each kinetic measurement was carried out in triplicate.

Above pH 7·0 a correction was made for the spontaneous rate of hydrolysis of the substrate, ($0{\cdot}39 \times 10^{-5}$ mole/l./hr). Reaction samples (2·5 ml) were prepared with a substrate concentration of $3{-}5 \times 10^{-4}$ M. Tannase solution (1, 2, 3, 4 and 5 μl) were added to five such samples and V_{max} determined for each one. A plot of the rate of *p*-nitrophenol production against the volume of tannase solution employed gave a straight line and extrapolation to zero vol. of tannase gave on the other coordinate the spontaneous rate of hydrolysis of the substrate at pH 7·1.

Synthesis of Substrates

3,4-Diphenylmethylenedioxy-5-methoxybenzoic acid. The acid was prepared by a procedure analogous to that of Jurd[18] and crystallized as plates from aq. acetone, m.p. 217° (lit.[16] m.p. 217°). (Found: C, 72·1; H, 4·7. Calc. for $C_{21}H_{16}O_5$: C, 72·4; H, 4·6%.) The acid chloride was prepared by the procedure of Mills[13] for the 5-acetoxy derivative and crystallized from light petroleum (b.p. 60–80°) as prisms, m.p. 111° (lit.[19] m.p. 109°). (Found: C, 68·9; H, 4·3; Cl, 9.7. Calc. for $C_{21}H_{15}O_4Cl$: C, 68·8; H, 4·1; Cl, 9·7%.)

o-, m-, *And* p-*nitrophenyl-3-acetoxy-4,5-diphenylmethylenedioxybenzoates.* 3-Acetoxy-4,5-diphenylmethylenedioxybenzoyl chloride (4·0 g) was dissolved in pyridine (20 ml) containing the nitrophenol (2·5 g) and stood at room temp. for 3 days. The solution was poured into ice-water and the product collected and crystallized from aq. acetone. The *o-nitrophenyl ester* was obtained as colourless plates, m.p. 185–186°. (Found: C, 67·8; H, 4·0; N, 2·9. $C_{28}H_{19}NO_8$ requires C, 67·6; H, 3·9; N, 2·8%), ν_{max} 1765 cm^{-1}, 1745 cm^{-1}. The m-*nitrophenyl ester* crystallized as colourless needles, m.p. 155–157°. (Found: C, 67·5; H, 3·8; N, 2·8. $C_{28}H_{19}NO_8$ requires C, 67·6; H, 3·9; N, 2·8%), ν_{max} 1775 cm^{-1}, 1735 cm^{-1}. The p-*nitrophenyl ester* was obtained as colourless needles, m.p. 173–175°. (Found: C, 67·6; H, 3·9; N, 2·9. $C_{28}H_{19}NO_8$ requires C, 67·6; H, 3·9; N, 2·8%), ν_{max} 1770 cm^{-1}, 1740 cm^{-1}.

o-, m-, *And* p-*nitrophenylgallates.* The appropriate ester above (1 g) was refluxed with aqueous acetic acid (75%, 30 ml) for 24 hr. The acetic acid was removed at 30° by rotatory evaporation and the residue treated under reflux with light petroleum (b.p. 60–80°, 4 × 50 ml). The residue was dissolved in EtOAc (20 ml) and washed with aqu. $NaHCO_3$ (3 × 20 ml) and water (20 ml). Removal of the EtOAc and crystallization from aq. acetone gave the nitrophenyl ester (0·3–0·4 g). The *ortho* isomer was obtained as a monohydrate which on heating at 120° and 0·05 mm gave the anhydrous compound. o-*Nitrophenylgallate* crystallized as yellow prisms, m.p. 180–182°. (Found: C, 53·3; H, 3·0; N, 4·7. $C_{13}H_{19}NO_7$ requires C, 53·6; H, 3·1; N, 4·8%), ν_{max} 1705 cm^{-1}. m-*Nitrophenylgallate* was obtained as yellow prisms, m.p. 226–227°. (Found: C, 53·9; H, 3·1; N, 4·8. $C_{13}H_{19}NO_7$ requires C, 53·6; H, 3·1; N, 4·8%) ν_{max} 1730 cm^{-1}. p-*Nitrophenylgallate* was obtained as yellow needles, m.p. 197–198°. (Found: C, 53·4; H, 3·3; N, 4·6. $C_{13}H_{19}NO_7$ requires C, 53·6; H, 3·1; N, 4·8%), ν_{max} 1720 cm^{-1} ν_{max}, 270 nm.

o-, m-, *And* p-*nitrophenyl-3,4-diphenylmethylenedioxy-5-methoxybenzoate.* The esters were prepared in a similar way to the acetoxy esters. The o-*nitrophenyl ester* crystallized as pale-yellow plates, m.p. 145–146°.

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(Found: C, 69·0; H, 4·1; N, 3·3. $C_{27}H_{19}NO_7$ requires C, 69·1; H, 4·1; N, 3·0%), ν_{max} 1730 cm^{-1}. The m-*nitrophenyl ester* was obtained as colourless needles, m.p. 176–177°. (Found: C, 69·0; H, 4·0; N, 3·1. $C_{27}H_{19}NO_7$ requires C, 69·1; H, 4·1; N, 3·0%), ν_{max} 1730 cm^{-1}. The p-*nitrophenyl ester* was obtained as colourless needles, m.p. 162–164°. (Found: C, 69·2; H, 4·3; N, 3·2. $C_{27}H_{19}NO_7$ requires C, 69·1; H, 4·1; N, 3·0%), ν_{max} 1735 cm^{-1}.

o-, m-, *And* p-*nitrophenyl*-3-O-*methylgallate*. The above esters were treated with acetic acid similarly to the acetoxy esters. o-*Nitrophenyl*-3-O-*methylgallate* crystallized as yellow prisms, m.p. 159–160°. (Found, after drying at 120° and 0·05 mm, C, 55·4; H, 3·7; N, 4·4. $C_{14}H_{11}NO_7$ requires C, 55·1; H, 3·6; N, 4·6%), ν_{max} 1725 cm^{-1}. m-*Nitrophenyl*-3-O-*methylgallate* crystallized as colourless needles, m.p. 139–140°. (Found: C, 55·3; H, 3·5; N, 4·3. $C_{14}H_{11}NO_7$ requires C, 55·1; H, 3·6; N, 4·6%), ν_{max} 1720 cm^{-1}. p-*Nitrophenyl*-3-O-*methylgallate* was obtained as colourless needles, m.p. 144–145°. (Found: C, 55·2; H, 3·6; N, 4·7. $C_{14}H_{11}NO_7$ requires C, 55·1; H, 3·6; N, 4·6%), ν_{max} 1720 cm^{-1}, λ_{max} 270 nm.