The Esterase and Depsidase Activities of Tannase

By E. HASLAM and J. E. STANGROOM
Department of Chemistry, University of Sheffield

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The esterase and depsidase activities of tannase have been examined by kinetic methods. Although the esterase/depsidase ratio of tannase may be varied by cultural methods and isolation procedures, evidence has been obtained to show that tannase, esterase and depsidase are enzymes with low specificities capable of hydrolysing both esters and depsides of gallic acid.

Previous work has shown the gallotannins to be almost exclusively poly-*O*-galloyl-D-glucose derivatives whose nature and complexity vary with the plant source (Haslam & Haworth, 1964). In all the gallotannins a certain proportion of the galloyl groups are bound in the form of *m*-depsides (I) but the disposition of these residues on the galloylglucose core is not yet proved. Several observations (G. Britton, E. Haslam & J. E. Stangroom, unpublished work), however, support the suggestion that the depsidically linked galloyl groups are not randomly distributed but form one polygalloyl chain of variable length linked to the carbohydrate nucleus at one specific position. In the light of this proposal a previous report (Toth & Barsony, 1943) that the gallotannin-decomposing enzyme tannase contains two separate enzymes, an esterase and a depsidase with specificities for methyl gallate (II) and *m*-digallic acid (III) ester linkages respectively, has been re-examined, since the possession of a pure tannase esterase should allow the isolation of a *m*-polygallic acid from the gallotannins if the above hypothesis regarding their structure is correct.

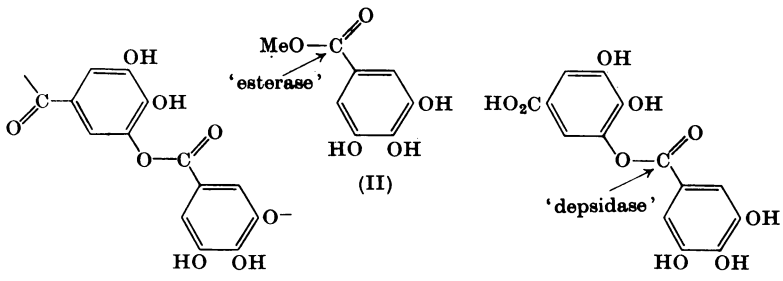
MATERIALS AND METHODS

Tannase. The enzyme was prepared by using a modified growth medium described by Knudson (1913). Two aqueous solutions, A (800ml.; containing $MgSO_4,5H_2O$, 0·5g., KH_2PO_4, 0·1g., KCl, 0·5g., and $NaNO_3$, 2·0g.) and B [200ml.; containing pure Chinese gallotannin, 20g. (Armitage *et al.* 1961) and $(NH_4)_2SO_4$, 1·0g.], were sterilized, the former by autoclaving and the latter by filtration through Carlson Steripads (grade E.K.O., iron-free). After aseptic transfer to penicillin flasks (A, 160ml.; B, 40ml.) the media were inoculated with *Aspergillus niger* 106 and cultured at 30° in the dark until a uniform black mat of sporangia covered the surface. The combined mycelia (20–25g.) were washed with distilled water and the tannase was extracted by one of the procedures outlined below (*a*, *b*, *c*). Media for the growth on methanolysed Chinese gallotannin were prepared analogously but with methyl gallate as carbon source; the ester (10·0g.) was added to solution A and both solutions were sterilized by autoclaving.

(*a*) Tannase acetone-dried powder. The mycelium (31g.) was extracted with pure acetone (500ml.) in a Waring Blendor at 5°, the suspension filtered, washed [acetone, acetone–ether (1:1, v/v) and ether, 400ml. each] and dried at 5°. The dried powder was shaken for 24hr. at 5° with 0·65M-NaCl solution (190ml.) and after filtration stored at 20°.

(*b*) Tannase aqueous extract. The mycelium (43g.) was blended in distilled water (430ml.) at 15° and the resultant suspension shaken for 24hr. at 15°. After filtration through acid-washed kieselguhr the solution was reduced to 85ml. by rotary evaporation at 25°, and stored at −20°.

(*c*) Tannase acetone precipitation. A portion of the extract prepared above (*b*) was treated at 5° with acetone (150ml.) containing ether (1ml.). The precipitate was



removed by centrifugation, redissolved in water (60ml.) and treated with acetone (150ml.) at 5° for 12hr. The precipitate was separated, dissolved in water (20ml.) and stored at 0°.

Assay of tannase activities. Tannase activities were determined at 37° with an autotitrator. The gallic acid produced by hydrolysis of portions of substrate solution (3ml., 12m-moles/ml.) by samples of tannase solution (0·1ml.) was continuously titrated against 0·01N-NaOH solution to maintain the reaction medium at pH6·0. The uptake of NaOH was continuously recorded against time and rates were expressed as the slope of the straight-line portion of the graph. All rate determinations were carried out in triplicate and were comparative rather than absolute; only rates observed during one continued series of runs were strictly comparable. The absence of phenol oxidases in the samples of tannase used was checked by incubating tannase solution with methyl gallate in a Warburg manometer at 37° in air. No uptake of oxygen could be demonstrated.

Chromatography of tannase on alumina. The procedure of Toth & Barsony (1943) was followed. Samples of alumina (untreated, acid-washed and neutral) were prepared according to Lederer & Lederer (1953). Portions (20ml.) of tannase solutions prepared by method (*b*) above were adjusted to pH6·6 with N-NaOH, passed over alumina (1·1g.) and the eluate was collected, dialysed against distilled water (4×1000ml.), freeze-dried and dissolved in 1mM-NaH_2PO_4 (5ml.) for assay.

Methyl gallate. This was prepared according to the method of Schopf & Winterhalder (1940).

m-*Digallic acid.* Commercial tannic acid (British Drug Houses Ltd., 100g.) was dissolved in 0·01N-HCl (1000ml.), heated at 100° for 6hr., cooled and continuously extracted with ether for 2 days. Removal of the ether gave a gum, which was dissolved in 0·5M-phosphate buffer (pH6·8, 1000ml.) and extracted with ethyl acetate (6×800ml.). The aqueous solution was treated with KCl (35g.), the pH adjusted to 2 with 10N-HCl and continuously extracted with ether (24hr.) to give an off-white powder (23·8g.) containing predominantly gallic acid and *m*-digallic acid (R_F values in 6% acetic acid, 0·52 and 0·25). Portions of this solid (1g.) were applied in acetone (25ml.) to 25 chromatoplates (27cm.^{2}, Solka Floc cellulose), which were developed in N-acetic acid. The chromatoplates were examined when dry under ultraviolet light and the slower-running blue fluorescent band (R_F 0·2–0·3) was cut out, combined and eluted with acetone. After filtration, the solvent was removed and the residual material redissolved in acetone (10ml.) and water added until the solution became cloudy, when it was left for 24hr. at 0°. The precipitate was centrifuged down and washed with a further quantity of water (10ml.). The supernatant liquids from the precipitation were combined and the solution rotary-evaporated at 25° until crystallization was incipient. The microcrystalline *m*-digallic acid (0·28g.) that separated had m.p. 258–261° [Crabtree, Haslam, Haworth, Mills & Stangroom (1965) give m.p. 260–261°].

Solutions for enzymic assay were prepared by dissolving the substrate (methyl gallate and *m*-digallic acid) in 1mM-NaH_2PO_4 or 0·6M-NaCl and the pH was adjusted to 5·8 with 0·5N-NaOH. Solutions of *m*-digallic acid prepared in this way showed decomposition after 5 days at 0° and only freshly prepared material was used in the enzymic assay.

Methanolysed Chinese gallotannin. Chinese gallotannin (100g.) was refluxed in methanol (1000ml.) for 7 days, when removal of the solvent and freeze-drying of the residue from *tert.*-butyl alcohol gave an off-white amorphous powder (95g.) of methanolysed Chinese gallotannin. The sample contained methyl gallate, β-1,2,3,4,6-penta-*O*-galloyl-D-glucose and 2,3,4,6-tetra-*O*-galloyl-D-glucose.

RESULTS AND DISCUSSION

The stability of the enzyme tannase under the conditions of assay and its non-inhibition by the products of reaction were checked qualitatively by allowing a sample of the enzyme to hydrolyse one of its typical substrates (II or III) to completion, when a further equal portion of substrate was added and the rate of hydrolysis once more recorded. For both substrates the two traces were identical and no inhibition of the enzyme was observed. The stability of the enzyme was further confirmed by showing that the activities of a given enzyme preparation stored at −20° and 37° for 12hr. were equal. In agreement with previous workers (Dyckerhoff & Armbruster, 1933) the rate of hydrolysis of a given portion of methyl gallate was directly proportional to the quantity of enzyme solution added, but attempted evaluation of the Michaelis constant K_m and V from typical tannase hydrolyses of methyl gallate by using the integrated form of the simple Michaelis–Menten equation (Dixon & Webb, 1964, p. 114) was unsatisfactory. A plot of

$$\frac{2{\cdot}303}{t}\cdot\log\left(\frac{S_0}{S_0-y}\right)$$

against y/t (where S_0 is the initial substrate concentration and S_0-y the substrate concentration at time t) gave, in all cases examined, a curve rather than a straight line and, although an elementary analysis revealed that this behaviour would result from the hydrolysis of the substrate by two enzymes having for the substrate different K_m and V values, this approach was not pursued.

A range of experimental evidence was obtained which showed that it was possible to vary the esterase/depsidase ratio of tannase [the activity against methyl gallate (II) compared with that against *m*-digallic acid (III)] within fairly wide limits (Table 1), supporting the original suggestion of Toth & Barsony (1943) that tannase contained distinct enzymes with esterase and depsidase specificities. Growth of *A. niger* on depside-free media, particularly methyl gallate, gave tannase with a marginal increase of the esterase/depsidase ratio compared with tannase grown on a gallotannin medium. Extraction procedures also affected the ratio, the highest esterase/depsidase figure being obtained by aqueous extraction of the mycelium

Table 1. *Esterase/depsidase ratio of tannase preparations*

A, B and C signify identical growth conditions of tannase preparations, esterase/depsidase (E/D) ratios being comparable within these groupings.

	Growth medium	Isolation procedure	Freeze-drying	Alumina chromatography	Ratio E/D
A	Chinese gallotannin	(*a*)	−	−	0·56
	Chinese gallotannin	(*b*)	−	−	0·95
	Chinese gallotannin	(*c*)	−	−	0·60
	Chinese gallotannin	(*a*)	+	−	0·50
B	Chinese gallotannin	(*b*)	−	−	1·06
	Methyl gallate	(*b*)	−	−	1·16
	Methanolysed gallotannin	(*b*)	−	−	1·10
C	Chinese gallotannin	(*b*)	+	−	0·64
	Chinese gallotannin	(*b*)	+	Untreated	0·56
	Chinese gallotannin	(*b*)	+	Acid-washed	0·62
	Chinese gallotannin	(*b*)	+	Neutral	0·69

Table 2. *Mixed-substrate hydrolysis*

E/D, Esterase/depsidase ratio.

Growth medium	Isolation procedure	Rates of hydrolysis* (II)	(III)	(II+III)	Ratio E/D
A Chinese gallotannin	(*b*)	1	1	0·85	1·0
B Chinese gallotannin	(*c*)	1	1·27	1·27	0·79
C Methyl gallate	(*b*)	1	0·85	0·89	1·17

* Rates of hydrolysis relative to the rate for methyl gallate (II).

(procedure *b*). Treatment with acetone at any stage substantially decreased the esterase activity, which was also deleteriously affected when freeze-drying procedures were used on the enzyme.

In earlier work (Haslam, Haworth, Jones & Rogers, 1961; Rogers 1959) tannase was fractionated by chromatography on Dowex 2 into four separate peaks with tannase esterase activity. In a repetition of this work each fraction was qualitatively observed to hydrolyse methyl gallate (II) and *m*-digallic acid (III), but, since freeze-drying techniques with their observed adverse effect on tannase esterase were necessary to concentrate the enzyme fractions, this approach for the concentration of tannase esterase was not applicable. Chromatography on alumina gave no marked change in the esterase/depsidase ratio and, contrary to the earlier reports of Toth & Barsony (1943), caused no separation of tannase esterase.

Application of the mixed-substrate method (Dixon & Webb, 1964, p. 84) to tannase led to the results shown in Table 2. In no case did the rate of hydrolysis of the mixed substrate (II plus III) approach the sum of the rates of hydrolysis of the individual substrates, but generally it was of the same order as the latter. Although the kinetic results of the mixed-substrate hydrolysis experiments may be interpreted by assuming that the two enzymes—esterase and depsidase—are reversibly inhibited by the substrates *m*-digallic acid (III) and methyl gallate (II) respectively, the more satisfactory explanation, in view of the known low specificities of other esterases, is that each of these enzymes is capable of hydrolysing both esters and depsides of gallic acid (II and III) and that each enzyme has only a relative specificity, one for esters the other for depsides. Experiments in which the esterase/depsidase ratio is lowered may then be interpreted as preferential removal or denaturation of the more labile enzyme with a relative esterase specificity. If these deductions on the nature of tannase are accepted then it seems improbable that the exhaustive fractionation of the crude enzyme mixture will yield an enzyme whose subsequent action on the gallotannins will lead to detailed information about their fine structure.

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