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THE INHIBITION OF ENZYMES BY TANNINS

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Abstract—A study has been made of the inhibition of β-glucosidase and other enzymes by tannic acid and a condensed tannin fraction from wattle. Tannic acid forms a 0·46:1 complex by weight with the glucosidase and complex formation can be reversed by a number of reagents, the best being non-ionic polymers or non-ionic and cationic detergents.

IT WAS suggested earlier that the loss of astringency which occurs during the ripening of certain edible fruits is due to increased polymerization of the tannins.[1] A number of inconsistencies were found, however, when attempts were made to substantiate this hypothesis by determining changes in the molecular size of tannins in fruit extracts by simple analytical techniques.[1] If astringency is assumed to be due to a cross-linking between tannins and proteins or glycoproteins in the mouth[2,3] it should be possible to measure the property by determining the binding power of tannins for selected proteins. Since this problem is of great importance in the leather industry a number of different techniques are available for use.[3] The classical hide-powder method for the determination of tannins does, of course, depend on this property, but gives no information as to the variation in the binding power of different tannins. A more useful approach is that described by Page,[4] in which the amount of precipitate formed with gelatin under strictly defined conditions is determined. A similar method, using casein, had been used earlier to examine the tannins of beer and wort.[5] Such methods however, suffer from the disadvantage that relatively large amounts of tannins are required.

It is known that tannins inhibit enzymes[6–16] and it appeared useful to exploit this fact to determine their binding power for protein. In this case residual protein, as enzyme, can be determined in small amounts with great precision, and thus it should be possible to develop a method for use with dilute tannin extracts. Besides giving a direct measurement of astringency,

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[11] W. L. PORTER and J. H. SCHWARTZ, *J. Food Sci.* **27**, 416 (1962).
[12] T. A. BELL, J. L. ETCHELLS, C. F. WILLIAMS and W. L. PORTER, *Botan. Gaz.* **123**, 220 (1962).
[13] W. MEJBAUM-KATZENELLENBOGEN, W. M. DOBRYSZYCHKA, J. JAWORSKA and B. MORAWIECKA, *Nature* **184**, 1799 (1959).
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[15] J. B. BIALE and R. E. YOUNG. *Vth Int. Congr. Biochem. (Abstracts) Ser. 15*, 312, Pergamon Press, Oxford (1961).
[16] M. HOLDEN, *J. Sci. Food Agric.* **10**, 691 (1959).

it was hoped that such measurements might indicate the importance the inhibition of enzyme by tannins might have in processes such as the ripening of fruits[15] or the "fermentation" of cacao.[16]

Most of the work which has been reported so far on enzyme inhibition by tannins has been carried out with systems involving a polymeric substrate (e.g. pectin[9,12]) and crude tannin extracts. The use of polymeric substrates make it difficult to interpret the results of many previous workers[6-12] since most of these substances are themselves capable of binding tannins. In such cases, therefore, the observed inhibition of an enzyme might be due partly to the formation of a substrate–tannin complex, which either is not attacked by the enzyme or may be a stronger inhibitor than the tannin alone. Furthermore, as will be shown later, many non-ionic polymers are capable of splitting tannin–protein complexes and thus regenerating enzyme activity, so that if the level of such activity is used as a measure of complex formation, the presence of polymeric substrates is highly undesirable. Finally it was felt that the successful concentration of enzymes and other proteins from dilute solutions by precipitation with tannins and subsequent regeneration of the protein in a completely unchanged state[13,14,17] gave promise that the reverse procedure could be used for the tannins. Obviously the presence of other potential tannin-complex formers would be a disadvantage.

Accordingly several different enzymes which catalyse the transformation of low-molecular-weight substrates were examined, and the most convenient from the point of view of availability and ease of assay were β-glucosidase (β-glucoside glucohydrolase) peroxidase (donor: H_2O_2 oxidoreductase), catalase (H_2O_2:H_2O_2 oxidoreductase), alcohol dehydrogenase (alcohol: NAD oxidoreductase) and lactic dehydrogenase (L-lactate: NAD oxidoreductase).

After a series of preliminary experiments, β-glucosidase was chosen for detailed study. The pure enzyme (from sweet almonds) is commercially available, easy to assay using the method described below, and whereas the substrate used (aesculin) does not form a complex with tannins, the enzyme is known to, since this procedure is used in its purification.[18] Although the majority of tannins in fruits are flavolans,[1] most of the work has been carried out using crystalline tannic acid (an octa- or nona-galloyl derivative of glucose[19]) since this compound, unlike the flavolans, can be readily obtained in a pure state.

It appeared preferable in every case to use a continuous method for the determination of enzyme activity so that the effect of the tannins on the initial rate could be observed. Although continuous methods for the measurement of β-glucosidase activity have been described, either artificial substrates are used,[20] or measurements are made at wavelengths at which tannin may interfere.[21] A new method has therefore been developed in which advantage is taken of the fact that the readily available glucoside aesculin yields an aglycone (aesculetin) which forms a coloured chelate with aluminium salts (λ_{max} 400 mμ). The reaction is carried out in acetate buffer at pH 4·8.

The general procedure for studying the inhibition of enzymes by tannins was to mix a solution of the two substances, leave for 30 min, centrifuge at 1500*g* for 5 min, and determine the amount of residual enzyme in the supernatant. Using this method it was found that the formation of a precipitate between β-glucosidase and tannic acid was dependent on pH,

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[21] B. H. J. HOFSTEE, *Arch. Biochem. Biophys.* **59**, 398 (1955).

ionic strength and the concentration of both protein and tannin.[3] Using both enzyme and tannic acid at a concentration of 1 mg/ml, precipitation was found to be virtually complete between pH 2·0 and 7·5. A similar range was found for tannin–gelatine precipitates by Davies *et al.*[22] Although less precipitate is formed at pH values lower than 2·0, the enzyme itself is denatured by such treatment. Above pH 7·5, the formation of a precipitate and the degree of inhibition is markedly dependent on the buffer used; at pH 8·0 in 2-amino-2-methyl-propane-1,3-diol, for example, no precipitate is formed and only 25 per cent inhibition is observed, whereas in phosphate there is a precipitate and 89 per cent inhibition.

The effect of ionic strength of the solution using phosphate at pH 7·0 is shown in Fig. 1. Here it can be seen that with strengths below 0·1 M, precipitation is incomplete as judged by the activity appearing in the supernatant. Even in very dilute buffers where no precipitation occurs the activity is still only 42 per cent of the control; this is in contrast to the activity of a resuspended precipitate (60 per cent of the control) and is presumably due to the excess of tannic acid present in this case (see below).

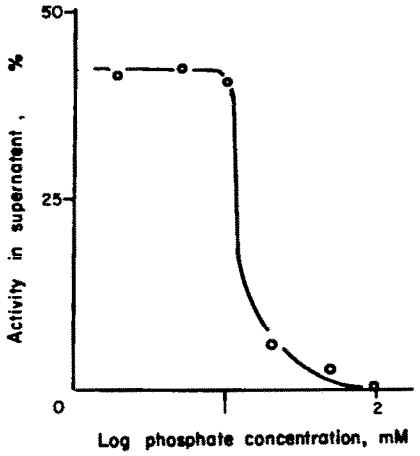

FIG. 1. EFFECT OF IONIC STRENGTH ON THE PRECIPITATION OF β-GLUCOSIDASE BY TANNIC ACID.
Ordinate: % activity in supernatant.
Abscissa: log phosphate concentration mM.

Having determined the parameters of pH and ionic strength which affect inhibition, the amount of tannic acid required to precipitate completely the enzyme and the amount bound to it in the complex was then determined. By adding incremental amounts of tannic acid to the enzyme in phosphate buffer 0·1 M, pH 7·0, it was found that 1 mg of β-glucosidase in 1·0 ml was completely precipitated by 0·75 mg of tannic acid; i.e. no activity was measurable in the supernatant. An independent assay of the amount of tannic acid in the supernatant showed that 0·460 mg of the acid was bound to the enzyme, and this amount remained constant when up to 5 mg of tannic acid were used. The ratio of 1:0·46 is near to that (1:0·5) found by Mejbaum-Katzenellenbogen and Dobryszychka[14] for rabbit muscle aldolase.

As mentioned above the precipitated complex of β-glucosidase and tannic acid surprisingly showed 60 per cent of the activity of the control when examined as a suspension. The Michaelis constant determined with the precipitate was the same as that of the pure enzyme ($K_m = 2{\cdot}22 \times 10^{-3}$ M) showing the inhibition to be non-competitive.

[22] R. I. DAVIES, C. B. COULSON and D. A. LEWIS, *Sci. Proc. Roy. Dublin Soc.* A1, 183 (1960).

It was stated earlier that one object of this work was to investigate the possibility of regenerating tannin–protein complexes so that the tannins may be obtained in a pure state. It has long been known that tannin–protein bonds may be broken by treatment with aqueous organic solvents or with urea.[3] Mejbaum-Katzenellenbogen and her co-workers found that caffeine, which forms an insoluble complex with many tannins, is a more satisfactory agent in that no denaturation of the proteins occurs.[13,14,17] More recently, in efforts to prevent loss of activity of enzymes[15] or particulate fractions[23] prepared from tannin-containing plants, several workers have described the use of non-ionic polymers. Hulme and Jones[24] have clearly shown that in the case of polyvinylpyrollidone (PVP) at least, this protective action is due to the formation of a complex between the polymer and the tannins. However, the activity of other reagents and their relative efficiency as tannin–protein splitters has not been investigated.

The effect of a large number of simple molecules, detergents and polymers has therefore been examined as reactivators of the β-glucosidase–tannic acid complex, that is for their

TABLE 1. STRUCTURE OF DETERGENTS USED FOR REACTIVATION

Trade name	Type	Structure	mol. wt.*
Ethylan	Non-ionic	*p*-Nonylphenylether of polyethyleneglycol	616
Lissapol N	Non-ionic	*p*-Isoöctylphenyl ether of polyethyleneglycol	646
Tween 80	Non-ionic	Dipolyethylene glycol ether of sorbitan monoöleate	604
Tengitol 08	Anionic	Sodium 7-ethyl,2-methyl-4-undecanol-sulphate	316
Manoxal OT	Anionic	Bis(2-ethylhexyl)sodium sulphosuccinate	444
Cetavlon	Cationic	Cetyltrimethyl ammonium bromide	364

* Approximate for the first three compounds.

ability to solubilize the enzyme from the complex in suspension in pH 6·0 phosphate buffer. With simple molecules, caffeine at 0·1 M (almost the limit of solubility) gave only 50 per cent activity; 0·1 M urea only 2 per cent, whereas 0·1 M borac acid gave 53 per cent recovery. Stronger solutions of urea (up to 5 M) gave appreciably higher recoveries, but in view of the success obtained with detergents, reactivation with simple compounds was not examined further.

Six detergents were examined over a wide range of concentration; three non-ionic, Ethylan, Lissapol N, and Tween 80; two anionic, Tergitol 08, and Manoxal OT; and one cationic, Cetavlon (Table 1). Only the non-ionic and cationic detergents proved to be effective (Fig. 2), Tween 80 being the best, giving complete reactivation at a concentration of less than 1 mg/ml (i.e. approximately equivalent to the amount of suspended complex). The failure of the anionic detergents to reactivate is noteworthy, since these compounds are capable of altering the tertiary structure of proteins.[25]

[23] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* **3**, 173 (1964).
[24] A. C. HULME and J. D. JONES, in *Enzyme Chemistry of Phenolic Compounds* (Edited by J. B. PRIDHAM), p. 97, Pergamon Press, Oxford (1963).
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A number of different high polymers were then tested including polyvinyl pyrollidone (PVP), polyethylene glycol (PEG), polyvinyl alcohol (PVA) and a number of alkylated celluloses. The two samples of PVP (average mol. wt. 11,000 and 25,000 respectively) and

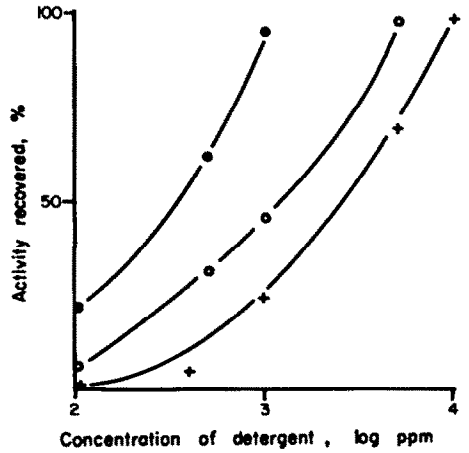

FIG. 2. REACTIVATION OF β-GLUCOSIDASE–TANNIC ACID COMPLEX BY DETERGENTS.
Ordinate: % activity recovered.
Abscissa: Concentration of detergent log ppm.
●——●, Tween 80; ○——○, Ethylan; +——+, Lissapol N.

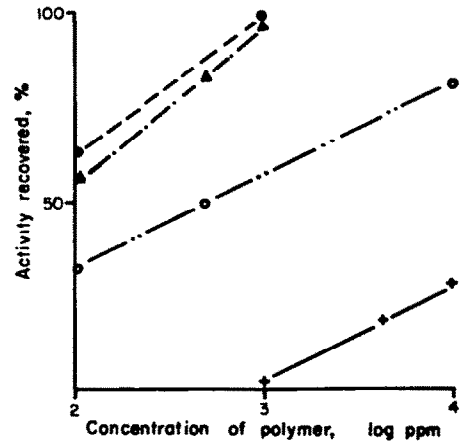

FIG. 3. REACTIVATION OF β-GLUCOSIDASE–TANNIC ACID COMPLEX BY POLYMERS.
Ordinate: % activity recovered.
Abscissa: Concentration of polymer log ppm.
●-----●, PVP; △—·—△, PEG 20M; ○—··—○, PEG 4000;
+——+, PEG 1000.

the highest molecular size PEG (average mol. wt. 20,000) were almost equally effective in restoring activity (Fig. 3) as was Tween 80. The low molecular size PEG samples (up to average mol. wt. 1000) were inactive, which is surprising in view of the success obtained with the non-ionic detergents all of which contain a short polyethylene glycol chain (Table 1 and Fig. 2). The curves relating concentration to reactivation for the active PEG type (Fig. 3)

are, however, different from that of the detergents (Fig. 2) and it would appear that the modes of action of the two groups are perhaps different. The other polymers were only tested at one concentration; 1 per cent PVA was about as effective (17 per cent) as PEG 1000 at the same concentration (Fig. 3); the different methyl celluloses (Methofas 450, 4500, and 20 M) all gave about 80–85 per cent reactivation at 0·5 per cent showing activity like that of PEG 4000. It is surprising that the increase in molecular size in this case appears to have no effect (cf. PEG samples). Finally, one sample of a carboxymethyl cellulose was examined and found, like the anionic detergents, to be inactive.

The effect of all these reactivation reagents strongly suggests that in the freshly prepared complex used in these experiments the protein and tannin are joined only by hydrogen bonds and coulombic forces. These would be broken by changes in pH, by dilution, or by changes in the ionic strength of the solution, leading to regeneration of the enzyme as was actually found. Furthermore the reactivation reagents can all act by either altering the

TABLE 2. REACTIVATION OF ENZYME–TANNIN COMPLEXES

Enzyme*	Tannin†	Protein/Tannin (mg/ml)	Activity of suspended precipitate	Activity in supernatant after treatment with						
				0·1 M Borate	0·1 M Caffeine	25% PEG 400	1% PVP	1% PVA	1% Me cellulose	25% Lissapol N
ADH	T	0·2/0·4	71	31	65	0‡	94	54	0	—
	W	0·2/0·4	24	0	30	0‡	35	24	0	24
LDH	T	0·5/1·0	0	0	2	3	65	6	66	—
	W	0·5/1·0	0	0	34	25	10	—	—	—
Peroxidase	T	0·4/1·0	59	14	27	29	23	35	42	31
	W	0·4/1·0	50	6	5	33	30	36	3	44
Catalase	T	0·1/1·0	58	0	24	19	79	26	0	—
	W	0·1/2·0	20	2	2	24	51	-	—	—
β-Glucosidase	T	1·0/2·0	52	40	50	45	74	71	22	66
	W	1·0/2·0	53	20	0	25	69	46	0	—

* ADH = alcohol dehydrogenase; LDH = lactate dehydrogenase.
† T = tannic acid; W = wattle tannin fraction.
‡ ADH is inhibited by PEG.
Anthocyanase did not form a precipitate with either tannin.

local charge on the tannin or the protein, or by competition for the tannin between the polymers used and the enzyme.

The results obtained in preliminary experiments with other enzymes, using both tannic acid and a condensed tannin fraction from wattle extract, are shown in Table 2. It can be seen that except for lactic dehydrogenase, the enzyme–tannin precipitates, like that of β-glucosidase and tannic acid, all show activity. With these enzyme–tannin complexes, borate proved to be a poorer reactivating agent than caffeine, and there were obvious differences in the reactivation of these complexes and that from β-glucosidase by the use of various polymers. The most important finding, however, is that the condensed tannin fraction appears to give more stable complexes than does tannic acid, since in only one case could more than half the activity be regained on reactivation. This points to the probability that links other than hydrogen bonds are involved in the formation of these complexes. The differences in the inhibition and reactivation of the various enzymes is also noteworthy, presumably resulting from differences in the fine structure of the proteins.

Although more work requires to be done, the results obtained give promise that the methods could be useful in determining changes in the binding power of tannins in fruit extracts. The extended range of reactivating reagents, especially the readily dialysable non-ionic and cationic detergents, should prove useful not only in extracting tannins but also as a means of protecting enzymes or particulate fractions from inhibition during their isolation from tannin-containing plants.

METHODS

Enzymes. Enzymes used were all commercially available except anthocyanase which was a gift from Rohm and Hass.

Enzyme Assays

β-Glucosidase. Aluminium chloride (30 μM) and aesculin (4 μM) in 3·0 ml of 0·1 M acetate buffer (pH 4·8) were placed in each of two 1 cm cells kept at 30° in a constant temperature cell housing of a recording spectrophotometer. The two cells were balanced at 385 mμ (the λ_{max} of the *difference* spectrum), and 50 μl of enzyme (up to 16 μg) added to the sample cell and well mixed. Under these conditions the hydrolysis follows first-order kinetics for at least 10 min, a rise in absorbancy of 0·01 being equivalent to the formation of 0·0045 μM of aesculetin. The maximum activity of this enzyme determined by a non-continuous method using phosphate–citrate buffer was found to be pH 5·2. These two acids however, interfere with the chelation of aesculetin by aluminium, and since aluminium forms a hydroxide above pH 5·6 in acetate buffer, pH 4·8 was chosen as a suitable compromise (rate is 94 per cent max.).

Other enzymes. Anthocyanase was assayed as for β-glucosidase. Alcohol dehydrogenase was assayed by following the reduction of NAD according to the method of Racker[26] but leaving out the gelatin from the buffer. Lactic acid dehydrogenase activity was determined with NADH and pyruvate,[27] and catalase by the spectrophotometric method of Beers and Sizer.[28] Peroxidase was assayed using *o*-dianisidine as a hydrogen donor.[29]

Tannins. Crystalline tannic acid free from gallic acid and chromatographically pure was presented by Dr. T. White. Commercial wattle extract (5·0 g) was extracted successively with 3 × 50 ml of boiling alcohol and then with water (50 ml). The water extract (10 ml) was added, with good stirring, to alcohol (100 ml) and the precipitate taken up in water (3 ml) and reprecipitated with alcohol. This precipitate was dissolved in water (5 ml) and dialysed overnight, and the extract (4·3 mg/ml) used after suitable dilution.

Reactivating reagents. Lissapol N, Tween 80, Tergetol 08, Manoxal OT and PVP (Kollidon 17, and Kollidon biologically tested) were commercial samples. Catavlon and the alkylated celluloses (Methofas 450, 4500, 20M, Cellofas A, Cellofas B 3500) were gifts from I.C.I. Ltd.; the polyethylene glycol (PEG) were gifts from Union Carbide Inc; Ethylan was a gift from the Lankro Chemical Co.; and an earlier used sample of PVP was a gift from British Oxygen Co.

Formation of Protein-Tannin Complexes

From β-glucosidase and tannic acid. Equal volumes of the enzyme 2 mg/ml and tannic acid 2 mg/ml in 0·1 M phosphate buffer at pH 7·0 were mixed, allowed to stand for 30 min

[26] E. Racker, *J. Biol. Chem.* **184**, 313 (1950).
[27] E. Racker, *J. Biol. Chem.* **196**, 347 (1952).
[28] R. F. Beers and I. W. Sizer, *J. Biol. Chem.* **195**, 133 (1952).
[29] *The Worthington Biochemical Corporation Manual No. 11*, p. 45, Freehold, New Jersey (1961).

and centrifuged for 5 min at 1500 *g*. The supernatant was discarded and the precipitate washed once with 0·1 M tris buffer at pH 6·0, and finally suspended in the same buffer at a concentration of 1 mg/ml with respect to the original enzyme.

From other enzymes. The enzymes and tannins were mixed in the concentrations shown in Table 2 to give 0·5 ml total and the precipitates recovered by centrifuging for 2 min at 20,000 *g*.

Treatment with Reactivating Reagents

β-Glucoside-tannic acid complex. The reactivating agent in 0·1 M tris buffer (0·2 ml pH 6·0) was added to the freshly prepared complex suspended in the same buffer (0·1 ml equivalent to 0·1 mg protein). The solution was left 30 min. centrifuged and the activity of the supernatant determined as before.

Other complexes. The precipitates obtained as described above were re-suspended in 0·2 ml of tris buffer (pH 6·0) containing the reactivating reagent and the whole left for 90 min. centrifuged and the activity of the supernatant determined.

Acknowledgements—We wish to thank Dr. T. White for a generous gift of pure tannic acid, and Messrs. Imperial Chemical Industries, British Oxygen Co., Union Carbide Inc., and Lankro Chemical Co. for gifts of the detergents and polymers used as reactivating agents. We also thank Messrs. Rohm and Hass for the gift of anthocyanase.