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Polyphenol–Protein Interactions

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The association of some natural and synthetic polyphenols with β-glucosidase was examined and some observations on the chemical nature of the complex were made.

It has long been recognized that the taste and stability of many edible fruits and fruit products is dependent on the type and concentration of astringents which are present. Astringency is due in part to the presence of tannins in the vegetative tissues, and the physiology of the astringency sensation is believed to result from the interaction of these natural polyphenols with salivary proteins and glycoproteins in the mouth (Bate-Smith, 1954; Goldstein & Swain, 1963, 1965). The chemical and biochemical changes underlying this phenomenon, and the analogous property of tannins to convert animal hides into leather, have, however, not been amenable to a comprehensive study, since until very recently the chemical structures of many of the natural vegetable tannins were not known and methods for their isolation in a chemically homogeneous form had not been realized (Haslam, 1966).

Various protein solutions have been proposed as test substances for the purposes of measuring vegetable tannins. Thus both gelatin (Jones, 1927) and casein (Handley, 1961) have been used, and Bate-Smith (1973) has utilized the proteins of haemolysed blood to determine the relative astringency of some tannins. In this work the association of some natural and synthetic tannins with the enzyme β-glucosidase (EC 3.2.1.21) has been examined in a quantitative manner and in the light of the experimental data some observations are made on the nature of the polyphenol–protein interaction.

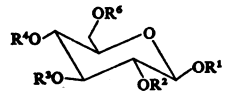
Experimental and results

β-Glucosidase from sweet almonds was used as a freeze-dried powder (BDH Chemicals Ltd., Poole, Dorset, U.K.; 4.4 units/mg). The precipitation of the enzyme was studied at pH5.0 by using the synthetic polyphenols (Scheme 1) (1–6; Armitage *et al.*, 1961; Schmidt, 1956), Chinese and Sumach gallotannins (7, $n = 0$, 1 and 2; Armitage *et al.*, 1961; Haslam, 1967), *Arctostaphylos* gallotannin (7, $n = 0$; Haslam & Britton, 1965), Turkish gallotannin (7, $n = 0$, 1 and 2, with positions 2 and 4 on the glucose ring variously unsubstituted; Armitage *et al.*, 1962), Tara gallotannin (8, $n = 0$ and 1; Haslam *et al.*, 1962) and two natural procyanidins B-2 (9a) and C-1 (9b), respectively a dimer and trimer of (−)-epicatechin (10) (Thompson *et al.*, 1972). The extent of precipitation of β-glucosidase was evaluated by measurement of the amount of enzyme that remained in solution after removal of the phenol–protein complex by using *p*-nitrophenyl β-D-glucoside as substrate. For each of the phenols several determinations of the amount of enzyme precipitated with increasing concentrations of polyphenol were carried out and a precipitation curve was plotted. From this plot values of the threshold concentration of phenol required for precipitation and the concentration required to precipitate a fixed amount of the enzyme (2mg, approx. 50% precipitation) were estimated. The values for 50% precipitation are tabulated (Table 1). Neither β-D-glucogallin (1) nor (−)-epicatechin (10) precipitated β-glucosidase under the conditions of assay.

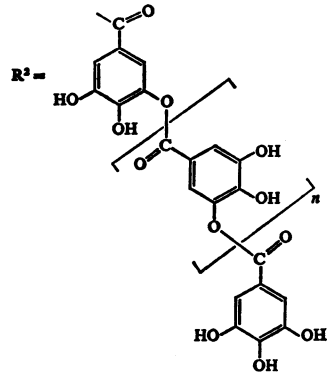
Control experiments were carried out for each of the polyphenols in which approximately 50% precipitation of the enzyme was achieved. The activity of the β-glucosidase was then determined before and after stirring (½h) with polyamide (Woelm; 0.1g) to remove residual polyphenol. In all cases the two activities showed a correlation of ±3% such as to suggest that the effects observed were due to enzyme precipitation and not to inhibition of enzyme activity by the presence of residual polyphenol in the enzyme solutions.

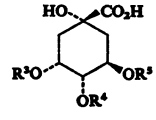
To a rapidly stirred solution of β-glucosidase (3–5mg) in sodium acetate buffer (0.2M, pH5.0; 2ml), acetate buffer (0.2M; respectively 1.5, 1.25, 1.0, 0.75, 0.5 or 0ml) and a solution of the phenol (respectively 0.5, 0.75, 1.0, 1.25, 1.5 or 2.0ml) in acetate buffer (0.2M, pH5.0) were added. After 15min the suspension was centrifuged to remove the precipitated phenol–protein complex and the concentration of the enzyme in the supernatant determined.

Enzyme solution (1.0ml) was added to a cuvette containing acetate buffer (0.2M, pH5.0; 11.0ml) and *p*-nitrophenyl β-D-glucoside solution (0.01M, in 3.0ml of 0.2M-acetate buffer, pH5.0) at 30°C. Samples (0.5ml) were taken after 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0min, added to Tris–HCl buffer (pH8.5;

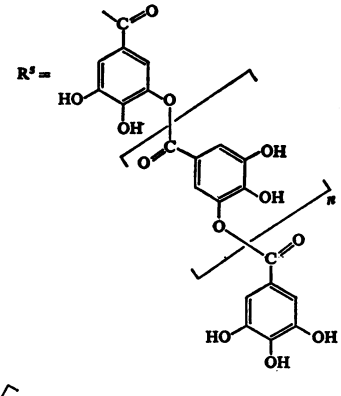


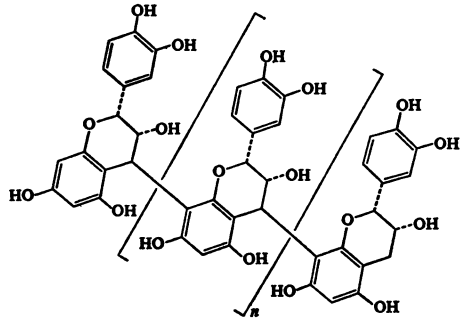
(1) $R^1 = -CO-C_6H_2(OH)_3$-3,4,5; R^2–$R^6 = H$
(2) $R^3 = R^6 = -CO-C_6H_2(OH)_3$-3,4,5; $R^1 = R^2 = R^4 = H$
(3) $R^1 = R^3 = R^6 = -CO-C_6H_2(OH)_3$-3,4,5; $R^2 = R^4 = H$
(4) $R^1 = H$; R^2–$R^6 = -CO-C_6H_2(OH)_3$-3,4,5
(5) $R^1 = Me$; R^2–$R^6 = -CO-C_6H_2(OH)_3$-3,4,5
(6) R^1–$R^6 = -CO-C_6H_2(OH)_3$-3,4,5
(7) $R^1 = R^3 = R^4 = R^6 = -CO-C_6H_2(OH)_3$-3,4,5



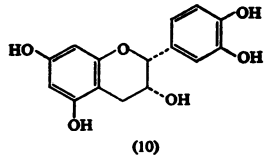


(8) $R^3 = R^4 = -CO-C_6H_2(OH)_3$-3,4,5

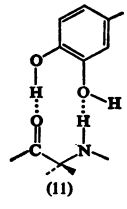




(9a) $n = 0$
(9b) $n = 1$



(10)



(11)

Table 1. *Precipitation of β-glucosidase by natural and synthetic polyphenols*

For details see the text.

	10^7 × Concn. for precipitation of β-glucosidase (50%, 2mg) (mol)	10^7 × Threshold concn. for precipitation (mol)	Mol.wt.
3,6-Digalloyl-D-glucose (2)	136	24	484
β-1,3,6-Trigalloyl-D-glucose (3)	33.9	7.5	636
2,3,4,6-Tetragalloyl-D-glucose (4)	13.6	2.8	788
β-Methyl tetragalloyl-D-glucoside (5)	16.4	3.4	802
β-Pentagalloyl-D-glucose (6)	4.4	0.7	940
Chinese/Sumach gallotannin (7, $n = 0$, 1 or 2)	4.3	0.6	1244*
Turkish gallotannin	6.0	0.9	1092†
Arctostaphylos gallotannin	6.3	0.9	1092†
Tara gallotannin (8)	20.6	3.5	794‡
Procyanidin B-2 (9a)	116	19.0	578
Procyanidin C-1 (9b)	31.0	4.6	866

* Based on an average heptagalloylglucose structure.
† Based on an average hexagalloylglucose structure.
‡ Based on an average tetragalloylquinic acid structure.

10.0ml) and the E_{420} was recorded. A plot of the *p*-nitrophenol formed against time gave a curve from which the initial reaction rate was determined.

Enzyme (protein) concentrations were obtained from a plot of initial reaction rate versus enzyme concentration. Four solutions were prepared containing acetate buffer (0.2M, pH5.0; 3.5, 3.0, 2.5 or 2.0ml respectively) and enzyme solution (1.5–2.5mg/ml, in 0.2M-acetate buffer, pH5.0; 0.5, 1.0, 1.5 or 2.0ml respectively). Samples (1.0ml) were taken as described above and the initial velocity of the enzyme-catalysed decomposition of *p*-nitrophenyl β-D-glucoside was determined. A plot of enzyme concentration versus initial velocity was then used to estimate the unknown enzyme concentrations.

Discussion

These results suggest that the β-penta-*O*-galloyl-D-glucose structure (6) represents the optimum configuration for complex-formation with proteins in the series di- → tri- → tetra- → penta-*O*-galloyl-D-glucose. Assuming an average molecular weight of 120000 for the enzyme β-glucosidase (Helferich & Kleinschmidt, 1965, 1968) the data suggest that for β-penta-*O*-galloyl-D-glucose the enzyme–polyphenol complex is formed in the ratio of approx. 1 molecule of enzyme to 20 of the phenol. This complex may be dissociated by repeated washing with acetone (Helferich *et al.*, 1932) and the enzyme activity may be restored to an extent greater than 75%. The natural hydrolysable tannins, such as Chinese and Sumach gallotannins (7, $n = 0$, 1 and 2), although based on the β-penta-*O*-galloyl-D-glucose structure, show no increase in complexing capacity on a mole-for-mole basis compared with compound (6). Further, in this series the increase in tanning ability is not linear with increasing galloylation (Table 1). The most reasonable rationalization of these observations is that the polyphenol–protein complex-formation which results in precipitation is caused by cross-linking of separate protein molecules by the phenol, and that the latter's tanning capacity, although broadly related to molecular size, is primarily dependent on the number of separate sites in the molecule able to associate with the protein. In the galloylglucose series these sites are presumably provided by each separate galloyl group and the capacity to cross-link requires a minimum of two such groups in the molecule. Thus for example Tara gallotannin (8, $n = 0$ and 1) has three and the natural gallotannins (7, $n = 0$, 1 and 2) and β-penta-*O*-galloyl-D-glucose (6) each possess five distinct sites of this type exposed on the surface of the molecule. However, β-D-glucogallin (1), with only one site, may associate with the protein but cannot engage in cross-linking. Analogously the experimental data indicate that the natural procyanidins B-2 (9a) and C-1 (9b) have respectively two and three sites for association. This observation points to the fact that the *o*-dihydroxyphenol groups in the natural tannin molecules are the sites for complex-formation and that isolated phenolic groups do not participate in the reaction. One way in which this specific association may take place is by hydrogen-bond formation with the keto-imide groups of the protein (Gustavson & Holm, 1952; Ellis & Pankhurst, 1954) in an arrangement analogous to that of the β-pleated sheet (11).

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