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THE EFFECT OF SOME AROMATIC COMPOUNDS ON PURE ENZYMES AND THEIR SUBSEQUENT REACTIVATION BY PVP AND TWEEN 80*

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Abstract—In plant tissues aromatic compounds are present which inactivate enzymes and render difficult their extraction and determination. The inhibition of the enzymes by aromatic compounds was investigated and reactivation by PVP and Tween 80 was successfully demonstrated.†

INTRODUCTION

A PARTICULARLY difficult problem in the field of plant biochemistry concerns the preparation of tissue extracts for a study of their enzyme content. Polyphenols, tannins, resins, terpenes, alkaloids and other potential enzyme inhibitors do not exhibit an inhibitory effect in the living cell because they are separated from the surrounding cytoplasm.[1] In plant extracts, however, they may inhibit many enzymatic systems.

The inhibition of spore germination and some enzymes (macerating enzyme, polygalacturonidase) in *Sclerotinia fructigena* by a number of phenolic and related compounds was shown earlier by Williams.[2] Hulme and Jones[3,4] provided evidence that tannin present in preparations of apple mitochondria lowered O_2-uptake and reduced the activity of several dehydrogenases and other enzymes; and that this inhibition could be in part reduced by addition of PVP to the extracting medium. Later, Goldstein and Swain[5] showed that tannin inhibition of alcohol-dehydrogenase, lactate dehydrogenase, peroxidase, catalase, β-glucosidase could be overcome by the addition of detergents and certain polymers. We have shown that MDH from *Pinus pinaster* is inhibited by tannin at low concentrations.[6]

The mechanism of action of such substances in reducing the enzyme activity is firstly by a reduction in the solubility of the enzyme proteins, by the formation of protein-phenolics complexes, and secondly by direct inhibition of the soluble enzymes.[3] We have shown that

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† In this paper the following abbreviations are used: MDH = malate dehydrogenase (E.C. 1.1.1.37); G6PDH = glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49); IDH = isocitrate dehydrogenase (E.C. 1.1.1.42); PVP = polyvinylpyrrolidone; TRA = triethanolamine hydrochloride; EDTA = ethylendiaminetetracetic acid, tetrasodium salt; NAD = nicotinamide-adenine dinucleotide; NADP = nicotinamide-adenine dinucleotide phosphate; OA = oxaloacetate; G-6-P = glucose-6-phosphate; Ic = isocitrate.

[1] T. SWAIN, in *Plant Biochemistry* (edited by J. BONNER and J. E. VARNER), p. 552, Academic Press, New York and London (1965).

[2] A. H. WILLIAMS, in *Enzyme Chemistry of Phenolic Compounds* (edited by J. B. PRIDHAM), p. 87, Pergamon Press, London (1963).

[3] A. C. HULME and J. D. JONES, *Enzyme Chemistry of Phenolic Compounds* (edited by J. B. PRIDHAM), p. 97, Pergamon Press, London (1963).

[4] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* 3, 173 (1964).

[5] J. L. GOLDSTEIN and T. SWAIN, *Phytochem.* 4, 185 (1965).

[6] A. GUERRITORE, P. VANNI, E. MASTRONUZZI and V. BACCARI, *Boll. Soc. Ital. Biol. Sper.* 41, 485 (1965).

by using PVP it is possible to demonstrate enzyme activity in the bark of mature trees, where, without PVP, no enzyme activity can be obtained.[7]

Subsequently, we have further improved the technique of extraction by incorporating in the medium Tween 80 at 1% concentration.[8] Goldstein and Swain had previously shown[5] that this compound with a preparation of β-glucosidase inhibited by tannin was the best of a series of ionic and non-ionic detergents studied to restore the enzyme activity. It permitted us to prepare active homogenates from needles of mature pine plants, which otherwise showed no enzyme activity.[8]

This paper reports data obtained on the reversal of inhibition by different aromatic compounds of three purified dehydrogenases (malate dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase) by adding PVP or Tween 80 to extraction medium.

RESULTS AND DISCUSSION

The results are shown in Figs. 1 and 2 and in Table 1. The effect of tannin is shown in Fig. 1; it must be pointed out that tannin at 5 μg/ml concentration completely inhibits the enzyme activity. Figure 2 shows the true course of an MDH assay and provides evidence for complete inhibition of MDH by abietic acid (final concentration 0·8 mM) and for reactivation by addition of PVP (final concentration 4·5 mg/ml) and Tween 80 (final concentration 1 mg/ml).

TABLE 1. EFFECT OF SOME AROMATIC COMPOUNDS ON MDH, IDH, G6PDH AND SUBSEQUENT REACTIVATION BY PVP AND TWEEN 80

Substance added*	Final concentration (mM)	Relative activity (as % of control)								
		MDH	IDH	G6PDH	MDH	IDH	G6PDH	MDH	IDH	G6PDH
					With PVP (4·5 mg/ml)			With Tween 80 (1 mg/ml)		
None	—	100	100	100	100	100	100	100	100	100
Tannic acid	0·07	0	0	0	67	84	67	81	99	41
Chlorogenic acid	0·5	20	0	0	20	0	0	20	0	0
K pyrogallate	0·8	0	0	21	71	69	60	0	0	29
Abietic acid	0·8	0	27	90	84	27	—	83	63	—

* The following compounds (added in the test cuvette at final concentration 5 mM) did not produce significant inhibition: *p*-hydroxybenzoic acid, catechol, thujic acid, *p*-aminobenzoic acid, salicylic acid, benzoic acid, gallic acid.

The results on the inhibiting effect of the aromatic compounds on MDH, IDH and G6PDH are shown in Table 1. From this data it is clear that tannic and chlorogenic acids are effective inhibitors of the three enzymes assayed. The tannic acid inhibition can be reversed both by PVP and by Tween 80. Inhibition by chlorogenic acid is not reversible.[9] The inhibition by potassium pyrogallate is reversed by PVP but not by Tween 80.

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[8] A. M. FIRENZUOLI, P. VANNI and E. MASTRONUZZI, *Boll. Soc. Ital. Biol. Sper.* **42**, 456 (1966).
[9] M. LIEBERMAN and J. B. BIALE, *Plant Physiol.* **31**, 420 (1956).

G6PDH seems to be almost insensitive to abietic acid, whereas MDH and IDH are inhibited by this compound. On the whole, the reactivation by PVP is nearly the same as that obtained with Tween 80.

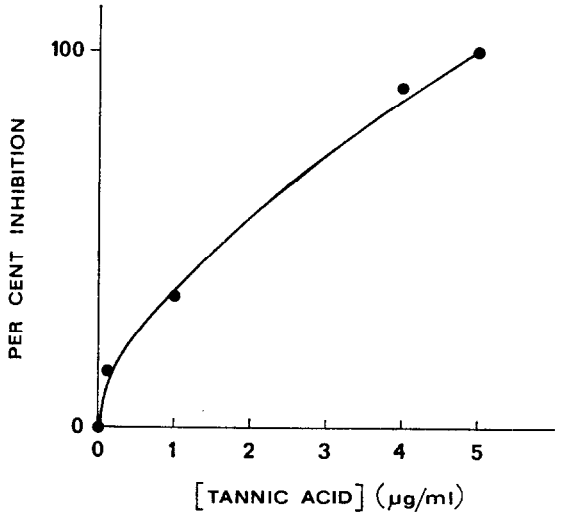

FIG. 1. INHIBITION OF PURE MDH BY DIFFERENT CONCENTRATIONS OF TANNIC ACID.

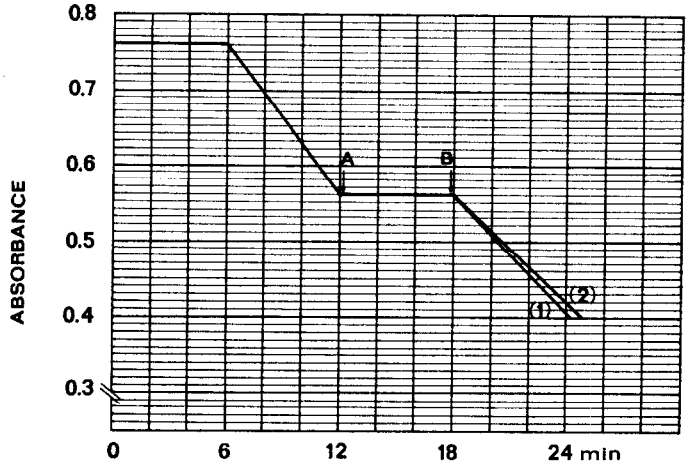

FIG. 2. INHIBITION OF PURE MDH AND REACTIVATION BY PVP AND TWEEN 80.

The absorptivity change of NADH oxidized to NAD during the experiment is shown in the graph. Addition of abietic acid (final concentration 0·8 mM) is indicated by the first arrow (A); the second arrow (B) indicates the successive addition of PVP (1) at final concentration of 4·5 mg/ml, or Tween 80 (2) at final concentration of 1 mg/ml.

In conclusion, a specific interaction between the enzymes and the inhibitors investigated seems very likely. It is very interesting to note that in our experiments the inhibition and the reactivation were not associated with protein precipitation. This tends to confirm the

hypothesis of the formation of soluble complexes between the enzyme and the inhibitor and between the latter and the reactivating agent.[10–12]

EXPERIMENTAL

Enzymic determinations were carried out by a continuous photometric recording of the extinction change between the reduced and oxidized forms of NAD and NADP at 366 nm, according to the method of Bücher *et al.*[13,14] using an Eppendorf recording photometer, operating with selected spectral lines from a mercury lamp. The photometer was equipped with a thermoregulated cell-holder and all tests were carried out at 25°. Enzyme activities were calculated as μmoles of transformed substrate per minute in the following standard reaction mixture. For MDH determination the standard reaction mixture was: TRA buffer pH 7·6 50 mM; EDTA 5 mM; OA 0·2 mM; NADH 0·15 mM; enzyme 5·5 mU/ml; final volume 1 ml. For IDH: TRA buffer pH 7·6 50 mM; EDTA 5 mM; $MgSO_4$ 8 mM; Ic 1·6 mM; NADP 0·5 mM; enzyme 14 mU/ml; final volume 1 ml. For G6PDH: TRA buffer pH 7·6 50 mM; EDTA 5 mM; G6P 1·8 mM; NADP 0·5 mM; enzyme 9 mU/ml; final volume 1 ml. In the thermoregulated cuvette, the reaction was started by addition of the substrate and allowed to proceed for 6 min, during which time the extinction change was recorded; then the potential inhibitor was added and the reaction permitted to proceed for a further 6 min. In cases of inhibition, the reactivation was tested (see Fig. 2) by adding PVP (final concentration 4·5 mg/ml) or Tween 80 (final concentration 1 mg/ml). All compounds assayed were used as aqueous solutions freshly prepared before every determination and adjusted to pH 7·6 with 0·2 M NaOH.

Chemicals Used

The enzymes used were pure preparations supplied by Boehringer uS., Mannhein. TRA, EDTA, NAD, NADH, NADP, oxaloacetate, isocitrate were Boehringer products; PVP (Kollidon 25, m.w. 24,000), Tween 80, chlorogenic acid and abietic acid were supplied by Fluka AG; G-6-P was supplied by Sigma Chemical Company; thujic acid was from K & K Laboratories (Plainview, New York); tannic acid was the pure product of Manetti & Roberts.

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