Phytochemistry, 1969, Vol. 8, pp. 1079 to 1088. Pergamon Press. Printed in England.

AN ENZYME FROM *EUCALYPTUS* WHICH CONVERTS CINNAMOYL TRIACETIC ACID INTO PINOSYLVIN

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(*Received* 12 *November* 1968)

Abstract—An enzyme catalysing the cyclization of cinnamoyl triacetic acid to form pinosylvin was isolated from *Eucalyptus sideroxylon* leaves and purified by Sephadex column chromatography. One enzyme preparation showed a 132-fold increase in specific activity and was fairly stable when stored as a freeze-dried preparation at 2–4°. The optimum pH of the reaction was at 8·2. Mg^{2+} at 2–10 mM concentration increased the rate of pinosylvin formation two and a half times, and Mn^{2+} and boron also stimulated the reaction rate to some extent. On the other hand, the enzyme was little affected by reagents such as *p*-chloromercuribenzoate but was strongly inhibited by KCN and cysteine, and also by mercury and iodine at high concentration. The function of this enzyme in plants is discussed.

INTRODUCTION

CHANGES in metabolism which result in marked differences in composition of secondary products can sometimes be readily achieved by the plant. Several chemovarieties of *Eucalyptus* spp. (Myrtaceae) have been found which contain stilbenes in the leaves in addition to the flavonoids and hydrolysable tannins normally present.[1] Both the normal and variant forms of some of these species have been found and in one case both forms grew within 100 m of each other. Morphologically the forms are indistinguishable and seedlings grown under different conditions have the same composition as the parents. Also, the sapwood of resistant *Pinus radiata* (Pinaceae) responds to infection by *Amylostereum* spp. by forming two stilbenes but no flavonoids, all of which are present in the heartwood but absent or in trace amounts in the sapwood.[2] The heartwood of a number of eucalypts (e.g. *E. astringens* and *E. polyanthemos*) contain stilbenes which are weakly present in the sapwood; both tissues contain flavonoids and hydrolysable tannins. An understanding of this change in direction of metabolism resulting in stilbene formation would assist the studies of the transformation of sapwood to heartwood and indicate the possibility of manipulation of the change with an improvement in disease resistance on the one hand and timber properties on the other.

Birch and Donovan[3] postulated that both flavonoids (by means of C-acylation) and stilbenes (by means of aldol condensation) are formed from a common precursor of the type $R—(CO.CH_2)_3CO_2H$ where R is Ph—CH=CH— or a related compound. It has been shown that acetate units form the A rings and shikimic acid and phenyl-propanoid compounds form the B rings of flavonoids[4–6] and stilbenes.[7–9] A study has been made of the relative

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[1] W. E. HILLIS, *Phytochem.* **5**, 541 (1966).
[2] W. E. HILLIS and T. INOUE, *Phytochem.* **7**, 13 (1968).
[3] A. J. BIRCH and F. W. DONOVAN, *Australian J. Chem.* **6**, 360 (1953).
[4] E. W. UNDERHILL, J. E. WATKIN and A. C. NEISH, *Can. J. Biochem. Physiol.* **35**, 219 (1957).
[5] T. A. GEISSMAN and T. SWAIN, *Chem. & Ind.* 984 (1957).
[6] S. SHIBATA and M. YAMAZAKI, *Pharm. Bull. Tokyo* **5**, 501 (1957).
[7] W. E. HILLIS and M. HASEGAWA, *Chem. Ind. (London)* 330 (1962).
[8] G. BILLEK and W. ZIEGLER, *Monatsch. Chem.* **93**, 1430 (1962).
[9] E. VON RUDLOFF and F. JORGENSEN, *Phytochem.* **2**, 297 (1963).

rates of biosynthesis of flavonols and stilbenes[10] but there has been no report on the biochemistry of the divergent pathways from the hypothetical precursor to the flavonoid or to the stilbene series. In the case of infected *P. radiata* sapwood (see above) it would appear that the pathway to flavonoids is blocked and the unstable precursor, which can cyclize under certain mild conditions without enzyme,[11] yields the components in the other pathway. The other cases could be due to formation of a new enzyme or a change in the amounts of cofactors or a greatly increased amount of non-phenolic precursors swamping the flavonoid pathway and spilling over into the stilbenoid pathway. Some of these aspects could be clarified if an enzyme capable of converting the precursor to stilbenes were found. This work reports the isolation of such an enzyme and its characterization. The enzyme has been classified as the cinnamoyl triacetic acid hydro-lyase (cyclizing-decarboxylase) belonging to class 4.2.1[12] and given the trivial name of pinosylvin synthase.

The preparation of acetone powders from woody or leathery leaf tissue has previously presented difficulties. In this work, a tissue-disintegrating device has been developed to give acetone powders of improved quality.

RESULTS

Enzyme Preparation

A unit of enzyme activity was defined as the amount of enzyme which catalyses the formation of 1 μM of pinosylvin in 1 min under the usual assay conditions. The activity based on protein concentration (specific activity) and the purity related to acetone powder were estimated.

Samples of acetone powder were prepared by two different methods. Acetone powder (A) was prepared in the special disintegrating machine (see Experimental) and acetone powder (B) by the usual method using a homogenizer. The colour of the acetone powder (A) was nearly creamy white, but (B) was coloured faint green even though acetone-washing was

TABLE 1. PURIFICATION OF PINOSYLVIN SYNTHASE FROM THE LEAVES OF *Eucalyptus sideroxylon*

Fraction	Yield (%) from fresh leaves	Units	Nitrogen content	Specific activity*	Purity
(a) *Leaves, batch* 1					
Acetone powder (B)	30·5	0·043	2·42	0·286	—
Acetone powder (A)	28·5	0·077	0·97	1·274	1
Dialysed extract	0·77	20·16	2·01	160·55	126·0
Sephadex G-50	0·75	25·06	3·98	168·45	132·2
(b) *Leaves, batch* 2					
Acetone powder (A)	32·4	0·01	1·85	0·09	—
Dialysed extract	2·2	11·54	2·63	70·19	—
Sephadex G-50	1·6	21·67	4·24	81·77	—
First Sephadex G-200	0·57	18·37	—	—	—
Second Sephadex G-200	0·55	16·21	4·16	62·35	—

* μmole pinosylvin produced per mg protein per minute under the usual conditions.

[10] W. E. HILLIS and K. ISOI, *Phytochem.* **4**, 905 (1965).
[11] T. M. HARRIS and R. L. CARNEY, *J. Am. Chem. Soc.* **88**, 2053 (1966).
[12] Anon., *Enzyme Nomenclature*, Elsevier, Amsterdam (1965).

repeated many times. Compared with (B), acetone powder (A) was shown to have a much higher enzyme activity (about 4·5 times) and a lower nitrogen content.

The enzyme in acetone powder (A), was extracted with 0·05 M tris-HCl buffer solution (pH 8·2) and the extract subjected to ultracentrifugation at high speed (105,000 *g*) followed by dialysis against distilled water. The enzyme solution was freeze-dried to a faint yellow-brown, amorphous powder with considerably increased activity (Table 1). A tris-buffer solution of the enzyme powder was passed through a Sephadex G-50 column and the absorbance at 280 nm of the 5-ml fractions was determined (Fig. 1). Fractions No. 5–68, which appeared to contain three overlapping fractions, were combined, dialysed against two changes of 2 l. of distilled water and freeze-dried. The activity of the brown, amorphous powder had a 132-fold greater activity than the acetone powder (Table 1) and this activity did not decrease

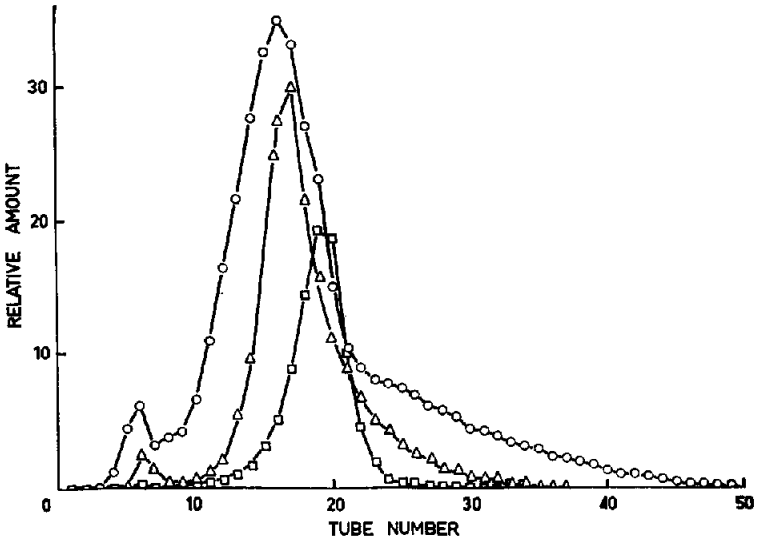

FIG. 1. FRACTIONATION OF PINOSYLVIN SYNTHASE PREPARATIONS ON SEPHADEX COLUMNS.

Batch 1, Table 1, from G-50, ○—○; Batch 2, first G-200, △—△; Batch 2, second G-200, □—□.

significantly when kept at 2–4° for several weeks. The absorption spectrum of the purified enzyme is shown in curve a, Fig. 2.

The effect of different purification procedures can be seen in the absorption spectra of the different enzyme preparations. The leaves chosen for the comparative study were at a more advanced stage of maturity and the acetone powder obtained had a lower activity than that from the first batch (Table 1). The powder was extracted with tris-HCl buffer, and the extract centrifuged and dialysed as before except that a lower centrifugal force (20,000 × *g*) was used for a three times longer period. The yield of enzyme was 2·5 times greater but the activity was about half (Table 1). The peaks at 312 and the absorption from 320 to 380 nm showed the presence of polyphenol impurities (curve b, Fig. 2). Fractionation of this material with a Sephadex G-50 column increased its activity (Table 1) and its purity (curve c, Fig. 2) by separating the impurities (curve d, Fig. 2) which are probably stilbenes[13] and flavonols

[13] W. E. HILLIS and N. ISHIKURA, *J. Chromatogr.* **32**, 323 (1968).

as the 368–372 nm peak shifted to 414 nm with aluminium chloride.[14] This enzyme preparation was further purified by fractionation through two Sephadex G-200 columns (Fig. 1). The group of tubes (No. 10–20) with the highest enzyme activities from the first column had a spectral curve e (Fig. 2) and from the second column (tubes 12–24) a spectral curve f (Fig. 2) showing that most of the polyphenolic impurities had been removed. Although the length of the purification procedure affected the enzyme activity (Table 1), it is evident that the

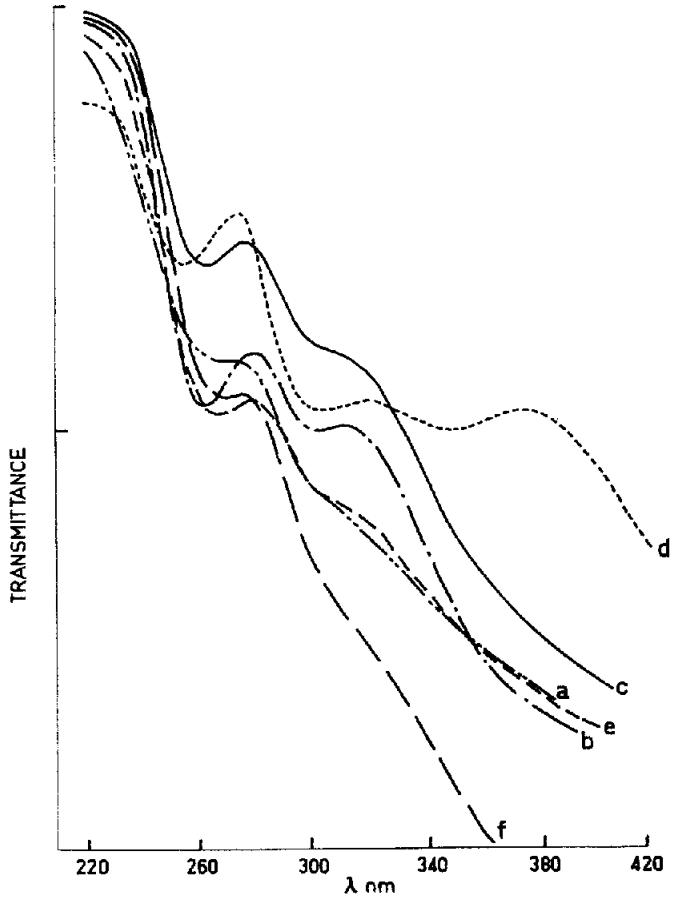

FIG. 2. ABSORPTION CURVES OF PINOSYLVIN SYNTHASE PREPARATIONS (SEE TEXT).
(a) Purified enzyme; (Table 1, Batch 1); (b) dialysed extract (Batch 2); (c) Sephadex G-50 preparation (Batch 2); (d) phenolic impurities removed by Sephadex treatment; (e) after Sephadex G-200 treatment (Batch 2); (f) after a second Sephadex G-200 treatment (Batch 2).

best purification procedure of this type would involve a high-speed centrifugation (105,000 × *g*) followed by two fractionations with Sephadex G-200.

Reactions Catalysed by the Enzyme

When the acetone powder and the enzyme preparations were incubated with cinnamoyl triacetic acid (9-phenyl-3,5,7-trioxo-8-nonenoic acid) (I), the latter was converted to *trans*-

[14] L. JURD, in *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), p. 107, Pergamon Press, Oxford (1962).

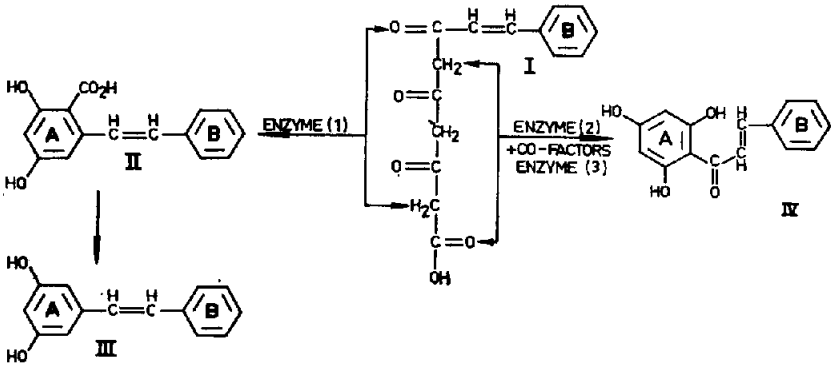

FIG. 3. THE CYCLIZATION OF CINNAMOYL TRIACETIC ACID (I) IN DIFFERENT WAYS TO GIVE A CHALCONE (IV) AND PINOSYLVIC ACID (II) AND PINOSYLVIN (III).

pinosylvin (III) (Fig. 3). Thin-layer chromatograms of the ether extract of the reaction mixture revealed a very small amount of a faint yellow compound in addition to the compounds (I) and (III), but there were no compounds corresponding to chalcones and flavonones of known type. The yellow compound is very unstable and the study of its properties is in progress. Results of blank experiments in which heat-inactivated enzyme (1 hr, 100°) or buffer alone were used showed that the cyclization of cinnamoyl triacetic acid did not occur under the experimental conditions used.

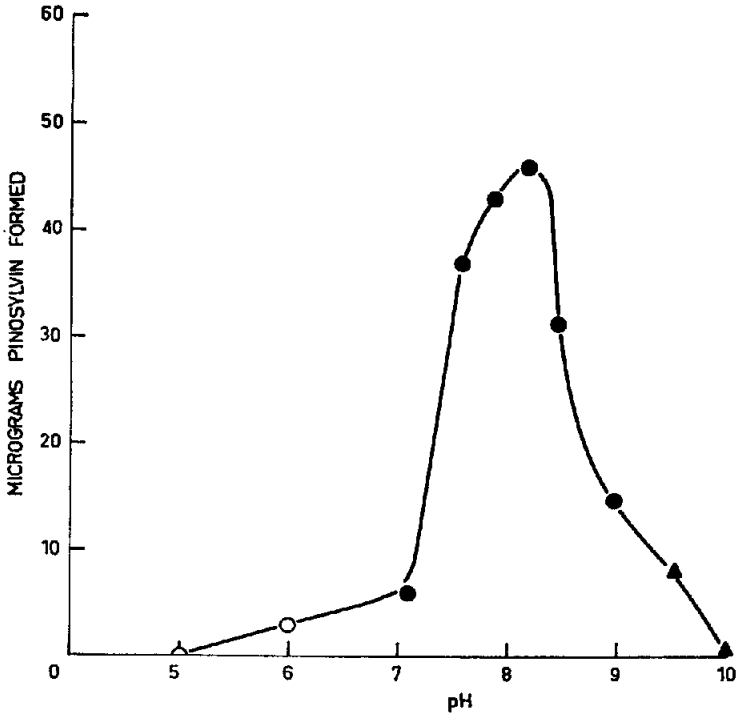

FIG. 4. THE EFFECT OF pH OF PINOSYLVIN SYNTHASE ACTIVITY. THE ENZYME PREPARATION (8·67 UNITS STRENGTH) WAS ASSAYED FOR 1 hr UNDER THE USUAL CONDITIONS.
The values were measured in M/10 potassium citrate buffer (○), M/20 tris-HCl buffer (●), and M/20 sodium borate buffer (▲).

Effect of pH

The pH optimum of the enzyme reaction was found to be 8·2, decreasing quite sharply to about 10% of optimum at pH 7·0 and pH 9·4.

Time Course of the Enzyme Reaction and Effect of the Enzyme Concentration

The formation of pinosylvin catalysed by 20·2 units of enzyme was linear for only 25 min and then decreased, and the assays were therefore conducted over this period. At low concentrations of purified enzyme (up to 1 mg) the rate of pinosylvin formation was proportional to the amount added to the reaction mixture, but this proportionality decreased considerably at higher concentration.

Stability of the Enzyme

The dried purified enzyme from the Sephadex G-50 column was stable for at least 1 month at 2–4°. However when the enzyme was dissolved in 0·05 M tris-HCl buffer solution for periods of up to 2 weeks at 20° ± 2° a 66·7 per cent loss of the activity took place.

Effect of Inhibitors and Activators

The effect of some reagents on the rate of reaction was studied and the results are shown in Tables 2 and 3. The addition of Mn^{2+} and Mg^{2+} at low concentrations was found to increase the amount of pinosylvin formed by two to two and a half times. The reaction was completely inhibited by a high concentration (1×10^{-2} M) of mercury ion (Table 2), strongly inhibited by potassium cyanide and cysteine and weakly inhibited by EDTA or diethyldithiocarbamate (Table 3). The latter effects suggest that the enzyme is dependent on some of the metals chelated by these inhibitors.

The reaction is not significantly inhibited by *p*-chloromercuribenzoate, mercaptoethanol or glutathione indicating that the action of the enzyme is not dependent on a sulphydryl group. On the other hand, the addition of iodine to the reaction mixture showed a high inhibitory effect on the reaction rate.

TABLE 2. EFFECT OF INORGANIC IONS OF THE ENZYMIC CONVERSION OF CINNAMOYL TRIACETIC ACID TO PINOSYLVIN

Addition	Final concentration (mMolar)	Effect (% of control)
$MgCl_2$	10	+150
$MgCl_2$	2	+153
$MnCl_2$†	10	+38
$MnCl_2$†	2	+88
H_3BO_3*	5	+71
$CuSO_4$*	10	+56
$CuSO_4$*	2	+57
$HgCl_2$	10	−100
$HgCl_2$	2	−28

The enzyme preparation (5·09 units strength) was assayed for 25 min under the usual conditions.

* Adjusted to pH 8·2 with NaOH. † Freshly prepared.

TABLE 3. EFFECT OF METAL-COMPLEXING AGENTS AND SULPHYDRYL GROUP REAGENTS ON THE ENZYMIC CONVERSION OF CINNAMOYL TRIACETIC ACID TO PINOSYLVIN

Addition	Final concentration (mMolar)	Effect (% of control)
KCN*	5	−76
KCN*	1	−73
Cysteine	20	−61
EDTA	5	−40
EDTA	1	−16
Diethyldithiocarbamate	5	−31
Diethyldithiocarbamate	1	−36
p-Chloromercuribenzoate	*ca.* 0·1 saturated	+26
Mercaptoethanol	10	−0–10
Glutathione	10	−10
Iodine	1	−84–90

The enzyme preparation (5·09 units strength) was assayed for 25 min under the usual conditions.

* Freshly prepared.

DISCUSSION

The postulated steps in the biosynthetic pathway from cinnamoyl triacetic acid (I) to stilbenes and flavonoids are shown in Fig. 3. The cyclization of the β-triketo acid to a stilbene derivative is mechanistically readily acceptable as an aldol condensation. This present work has provided evidence that under the conditions used this reaction requires enzyme mediation and that the acetone powder from *Eucalyptus sideroxylon* (stilbenoid chemovar) leaves can catalyse the change to pinosylvin (III) without the aid of co-factors.

It is possible that an additional enzyme is necessary to decarboxylate pinosylvic acid (II) to form pinosylvin. However, both reactions took place with a purified pinosylvin synthase and, in addition, there was no evidence of a lag phase and the rate of pinosylvin formation was linear for at least 25 min and was proportional to the amount of enzyme used. Consequently decarboxylation must proceed rapidly after cyclization without the need of a second enzyme. Earlier work showed stilbenes to be formed more slowly than flavonoids and it was considered that decarboxylation could be a rate-determining step;[10] this present work shows that some other factor must be responsible. It is noteworthy that the only stilbene carboxylic acids occurring naturally (hydrangeic and phyllodulcic acids) lack a hydroxyl *para* to the carboxyl group. By analogy with the 5-deoxyflavonoids it is probable the relevant hydroxyl is removed before cyclization of the A ring,[15] so that it has no opportunity to promote decarboxylation.

Because of the above observations we have classified the pinosylvin synthase enzyme as the cinnamoyl triacetic acid hydro-lyase (cyclizing-decarboxylase) Class 4.2.1.

Pinosylvin synthase showed maximum reaction activity at pH 8·2 and low concentrations of Mg^{2+} and Mn^{2+} were shown to stimulate the activity to a high degree. The enzyme was still active without the addition of these metal ions, although it was not rigorously purified to remove metals. The enzyme was found to be inhibited by potassium cyanide and cysteine which indicates it could be a metalloprotein. The lack of response to reagents such as

[15] T. SWAIN and E. C. BATE-SMITH, in *Comparative Biochemistry* (edited by M. FLORKIN and H. S. MASON) Vol. 3A, p. 755, Academic Press, New York (1962).

p-chloromercuribenzoate showed that the activity of the enzyme was not dependent on the sulphydryl group. However, the addition of iodine, a thiol-oxidizing reagent, strongly inhibited the reaction rate. It has been shown that thiol groups of enzyme are not always in the active centre, for example thiol groups of aldolase can be destroyed without loss of activity.[16] The inhibitory effect of iodine and also mercury, might, therefore, have some other implication rather than thiol oxidation, for example mercury could be acting as a protein precipitant.

It is notable that the rate of formation of pinosylvin decreases after 25 min. Losses due to oxidation of pinosylvin under the conditions used would probably be insignificant. The cause is more likely to be due to deactivation of the enzyme by pinosylvin in a process which the purification studies indicate to be reversible.

The batches of pinosylvin synthase were obtained from leaves of *E. sideroxylon*. Among the polyphenols present in the leaves of this species are the glucosides of the 3,4′,5-trihydroxy-, 3,3′,5-trihydroxy-4-methoxy- and 3,3′,4′,5-tetrahydroxy-stilbenes (but no pinosylvin) as well as glycosides of kaempferol, dihydroxykaempferol and quercetin and derivatives of gallic acid.[10] The present work shows pinosylvin to be the only stilbene produced from cinnamoyl triacetic acid by an unpurified acetone powder from these leaves when co-factors were not added to the medium. Thus cyclization to stilbene can occur independently of the hydroxyl pattern in ring B. Furthermore it is apparent that co-factors or special conditions are necessary for hydroxylation of ring B or, alternatively, prior hydroxylation at 4′ is required before further hydroxylation takes place. Less than 10 per cent of another polyphenol (λ_{max} 296) was formed from (I) by the unpurified powder but no evidence of chalcone was observed. Consequently either another cyclizing enzyme (No. 2, Fig. 3) is required for flavonoid formation, or alternatively co-factors are required for the pinosylvin synthase (No. 1, Fig. 3) to catalyse this reaction. Incubation of the triacetic acid with purified enzyme, ATP and co-enzyme A failed to produce flavonoid so that another enzyme could be required for the formation of the co-enzyme A ester.

The tissue-disintegrating machine which crushed the leaves between rollers (see Experimental) gave acetone powders with higher activity than those produced by the usual type of blender using rapidly rotating cutting blades. Preliminary trials indicate the machine is superior to a blender for the treatment of tougher tissues such as wood sections.

EXPERIMENTAL

Plant Material

Mature leaves of *Eucalyptus sideroxylon* (stilbenoid chemovar) were obtained from epicormic shoots during winter and early spring and the fresh leaf blades (separated from petiole) were processed within 4 hr of removal of the shoot from the tree.

Enzyme Preparation

All manipulations during enzyme preparation were carried out in a cold room at 2–4° and all preparations were stored in tightly stoppered containers at 2–4°.

Acetone powder (A). The leaves (40 g) were soaked *in vacuo* in cold acetone (250 ml, 2–4°) for 20 min and then the swollen material was crushed between two rotating stainless-steel rolls of the tissue-disintegrating machine (see later). The crushed material was suction-filtered and the residual powder was washed with 200 ml of acetone twice and then dried for 1 hr in vacuum. The yield of acetone powder was 11·40 g (Table 1).

In batch 2 the leaves (50 g) yielded 16·20 g of acetone powder (Table 1).

Acetone powder (B). The leaves (10 g) were chopped to a small size and soaked in three changes of cold acetone (120 ml, 2–4°) for 10 min and then disintegrated in cold acetone (70 ml, −30–40°) in a M.S.E.

[16] M. DIXON and E. C. WEBB, in *Enzymes* (2nd edition), p. 466, Longmans, London (1964).

Homogenizer for 6 min. The homogenate was suction-filtered, washed with 50 ml of cold acetone and dried in vacuum, yield 3·05 g (Table 1).

Extraction of Enzyme from Acetone Powder and Purification by Dialysis

10 g of acetone powder (A) from the first batch of leaves were suspended in 250 ml of 0·05 M tris-HCl buffer (pH 8·2) containing 0·05 M mercaptoethanol and stirred in a flask for 1 hr. The suspension was squeezed through a nylon cloth and centrifuged at 105,000 *g* for 10 min at 2–4°. The supernatant solution was dialysed against 3 × 2·5 l. of distilled water for 48 hr and freeze-dried, yield 270 mg (Table 1).

12 g of acetone powder (A) from the second batch of leaves was extracted with the tris-HCl buffer as above and then centrifuged at 20,000 *g* for 30 min. The supernatant (about 250 ml) was dialysed against 2 × 4 l. of distilled water for 24 hr and freeze-dried, yield 814 mg (Table 1; curve b, Fig. 2).

Fractionation with Sephadex Columns

Sephadex G-50. The freeze-dried enzyme (200 mg) obtained from the first batch of leaves was dissolved in 4 ml of 0·05 M tris-HCl buffer (pH 8·2) and placed on a Sephadex G-50 column (30 × 2·5 cm). The column was developed with the buffer and 5-ml fractions collected. The absorptivity (280 nm) of each fraction was determined showing three overlapping regions with the main peak at tube No. 30 and two minor peaks at tubes No. 12 and 58. Enzyme activity was found throughout these regions, although it was highest in the middle portion. Tubes No. 5–68 were combined, dialysed against two changes of 2 l. of distilled water for 24 hr and freeze-dried. Yield of dried residue was 195 mg (Table 1).

The freeze-dried enzyme (600 mg) from the second batch of leaves was fractionated as above but 10-ml fractions were collected. Following the determination of the absorptivity at 280 nm (Fig. 1) tubes No. 7–26 were combined, dialysed against two changes of 4 l. of distilled water for 24 hr and freeze-dried, yield 430 mg (Table 1, curve c, Fig. 2). The absorption spectra of tubes 27–40 is shown in curve d, Fig. 2, and the peak at 370 nm was shifted to 414 nm on addition of aluminium chloride.

Sephadex G-200. Partially purified enzyme (350 mg) from the second batch of leaves (after fractionation on a G-50 column) was dissolved in 4 ml of 0·05 M tris-HCl buffer (pH 8·2) and passed through a G-200 column as above and 10-ml fractions collected. On the basis of absorptivity at 280 nm the tubes were combined into three groups (Fig. 1) which were dialysed and freeze-dried: a, tubes 5–8, yield 60·8 mg; b, tubes 10–20, yield 126 mg; c, tubes 21–32, yield 40 mg. Group "b" had the highest activity (Table 1) and gave absorption curve e (Fig. 2). This fraction (100 mg) was again fractionated through G-200 and tubes 12–24 combined, dialysed and freeze-dried, yield 97 mg (Table 1; curve f, Fig. 2).

Enzyme Assay

Trans-pinosylvin was identified as the product of the enzyme reaction by its spectral and chromatographic properties.[13] The reaction mixture contained the enzyme preparation (200 mg acetone powder or 1 mg purified enzyme) and 1·25 μmoles of cinnamoyl triacetic acid (5–10 times theoretical requirement) in a final volume of 4 ml of 0·05 M tris-HCl buffer, pH 8·2. The reaction mixture was incubated without shaking for 25 min in a test tube at 30°. The reaction was stopped by the addition of dil. HCl to pH 5. The acidified reaction mixture was extracted repeatedly with ether, the extract dried (Na_2SO_4), evaporated to dryness in vacuum and the residue resolved on a chromatoplate (75 μ thickness) of Silica Gel GF 254 (E. Merck, A. G. Darmstadt) using MeOH–$CHCl_3$–petrol (90–120°) (2:4:7, v/v) as solvent. After development, the band of pinosylvin was detected by u.v. light, extracted rapidly with acetone before the plate dried and then evaporated to dryness in vacuum. The residue was dissolved in a known volume of ethanol and the absorbance at 312 nm was determined. The amount of pinosylvin was determined by comparison with the standard curve of the authentic compound which was linear in the range of 0·2–1·0 mg/100 ml.

Estimation of Protein

Was determined by Kjeldahl's method by the Australian Microanalytical Service.

Other Polyphenol Produced

A faint yellow compound was produced in very small amount by acetone powder. It had R_f 0·20 (TLC with MeOH/$CHCl_3$/petrol, 2:4:7) and a sharp absorption peak at 296 nm in ethanol alone or with NaOEt, and 300 nm with a shoulder at 308 nm after the addition of aluminium chloride and of boric acid and sodium acetate.

Reagents

We are very grateful to Dr. T. M. Harris of Vanderbilt University, U.S.A., who kindly donated a sample of cinnamoyl triacetic acid (m.p. 123° dec.). The pinosylvin was isolated from the heartwood of *Pinus radiata*. All of the other reagents used were commercial analytical grade.

Tissue Disintegrating Machine

The machine, which is a development of one used by Dr. J. D. Jones (personal communication), consisted of two stainless-steel rolls 4 in. long and 1·5 in. dia. with matt finished surfaces, held tightly together in a frame and rotated between single row ball-bearings. A hollow scraping device was held by a spring against the underside of the rolls, and through a number of fine holes in the device a spray of acetone was continuously directed on to the bottom of the rolls and the crushed leaves. The machine was kept in the cold room (2–4°) at all times and the acetone-swollen leaves were passed one at a time through the rolls and acetone was sprayed on to the top part of the rolls during crushing. The leaves were disintegrated to a colourless powder of very small particle size within a few sec. From time to time the scraping device was lowered and the partly washed powder washed with acetone into the collecting tray underneath the rolls. An almost quantitative recovery of material was achieved. We wish to acknowledge the help of Mr. K. G. Murray during the development and building of the machine.