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CONVERSION OF *p*-COUMARIC ACID TO *p*-HYDROXYBENZOIC ACID BY CELL FREE EXTRACTS OF POTATO TUBERS AND *POLYPORUS HISPIDUS*

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Key Word Index—*Solanum tuberosum*; Solanaceae; *Polyporus hispidus*; Polyporaceae; *p*-coumaric acid; *p*-hydroxybenzoic acid; non-oxidative enzyme reaction mechanism.

Abstract—The conversion of *p*-coumaric acid to *p*-hydroxybenzoic acid was demonstrated *in vitro* in both potato tuber and *P. hispidus*. The mechanism of the enzyme system is non-oxidative. This is the first report of a cell free system which is capable of converting a C_6-C_3 acid to the corresponding C_6-C_1 derivative from a fungus.

It has been known for some time that both higher plants and fungi are capable of the conversion of C_6-C_3 acids to C_6-C_1 derivatives *in vivo* [1–3]. However until recently nothing was known of the enzymology of the reactions. Zenk [4] has proposed a mechanism similar to the β-oxidation of fatty acids and the studies of Alibert *et al.* [5,6] are in accordance with this hypothesis (Fig. 1a). Conversely the production of *p*-hydroxybenzoic acid from *p*-coumaric acid by preparations of isolated glyoxysomes from *Ricinus communis* [7] appears to have no requirement for CoA and may be similar to the system described in *Pseudomonas acidivorans* [8] (Fig. 1b). In this paper we report the isolation of an apparently non-oxidative side-chain shortening enzyme system from potato tuber and evidence for an analogous system in the basidiomycete *Polyporus hispidus*.

The pH optimum of the enzyme system in potato slices showed a broad specificity with peak activity at pH 8·5. The system was very unstable and heat-labile, complete loss in activity being observed on storage at 4° for 24 hr and incubation of the reaction mixtures at 40°. No means of stabilising the enzymes has been found. No activity was detected in extracts from freshly-sliced tubers and there was no effect of light on the slices incubated for 24 hr.

The products of the chain-shortening enzymes were *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and, in the presence of mercaptoethanol, *p*-hydroxybenzyl alcohol. The latter was a product of the aldehyde; incubation of *p*-hydroxybenzaldehyde with a solution of 5 mM mercaptoethanol in KH_2PO_4 buffer at pH 8·5 and 30° produced an identical result.

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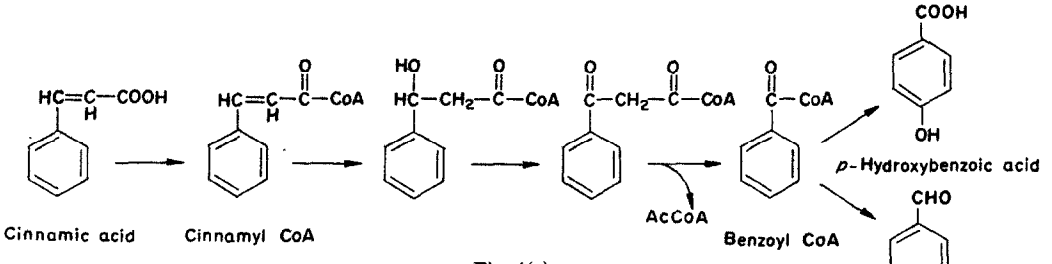

Fig. 1(a)

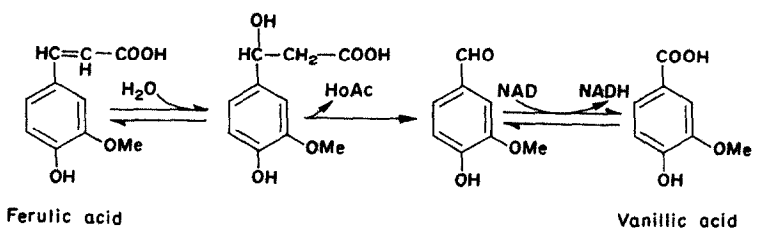

Fig. 1(b)

Table 1. Activity of cell free extracts in the conversion of *p*-coumaric acid to *p*-hydroxybenzoic acid

Enzyme source	Reaction mixture	Specific activity (nmol *p*-hydroxybenzoic acid/hr/mg protein)
Potato tuber slices	Complete (+ CoA, + ATP + NAD)	0·60
	− NAD	0·50
	− CoA/ATP	0·70
	− CoA/ATP, − NAD	0·56
	− enzyme	0·00
Polyporus hispidus	+ NAD	1·20
	− NAD	0·00
	− enzyme	0·00

The conversion of *p*-coumaric to *p*-hydroxybenzoic acid was independent of additional CoA, ATP and NAD (Table 1). This suggests that the reaction proceeds by a mechanism similar to Fig. 1b, i.e. non-oxidatively. However the step from *p*-hydroxybenzaldehyde to *p*-hydroxybenzoic acid should require NAD. Consequently the activity of *p*-hydroxybenzaldehyde oxidase in the enzyme extract was investigated separately by supplying the aldehyde as substrate. It was found that the reaction was not NAD-dependent, possibly due to close association of the enzyme with endogenous co-factor. This may explain the conversion of *p*-coumaric acid through the C_6-C_1 aldehyde to *p*-hydroxybenzoic acid without the necessity for additional NAD. The independence of the reaction on CoA and ATP suggests a non-oxidative mechanism for the production of *p*-hydroxybenzaldehyde. However this is not conclusive evidence since it is possible (although unlikely) that these factors might be limiting for a similar reason to that above. In order to conclusively demonstrate a non-oxidative reaction it will be necessary to separate the (presumably) two enzyme activities. However the intermediate corresponding to that in Fig. 1b would be 4,β-dihydroxyphenylpropionic acid for which there appears to be no obvious synthesis [8]. The production of this compound (or its isolation from an enzymatic reaction) seems to be the major problem limiting our progress.

Acetate was detected in the reaction mixture by GLC and was found to be produced in approximately equal quantities to *p*-hydroxybenzoic acid. This suggests that the assay system employed gives a reasonable estimate of the activity of the side-chain shortening enzymes since, in theory, acetate and *p*-hydroxybenzaldehyde should be produced in equal quantities (by either reaction mechanism). It appears therefore that the diversion of small amounts of *p*-hydroxybenzaldehyde into *p*-hydroxybenzyl alcohol and the incomplete conversion of aldehyde to acid (*p*-hydroxybenzaldehyde can be detected in small amounts in the reaction mixture) are of little importance.

Studies on *P. hispidus* resulted in the isolation of an enzyme system analogous to that in potato tuber. However in this case complete dependence on NAD was observed (Table 1). This may be due to the properties of the *p*-hydroxybenzaldehyde oxidase which was found to be completely dependent on NAD when assayed separately. It remains to be seen whether the production of *p*-hydroxybenzaldehyde proceeds oxidatively or non-oxidatively in this organism. To our knowledge this is the first report of an *in vitro* enzyme system from a fungus with this capability. We are aware, however, of similar studies proceeding elsewhere (Dr. P. V. Subba Rao, Indian Institute of Science, Bangalore, India, personal communication).

EXPERIMENTAL

Slices of potato tuber were prepared as previously described [9] and incubated at 25°C for 24 hr before extraction in incubators equipped with 'cool white' fluorescent tubes. *Polyporus hispidus* (UBC513) was cultured as described [10]. Cultures were maintained in incubators as above. Light grown mycelium was produced from cultures given 0·5 hr light/day. The mycelium was harvested at day 14.

Preparation of cell-free samples–potato. 100 g fr. wt slices were blended quickly in 200 ml KH_2PO_4 buffer, pH 7·5, 0·2 M containing 5 mM mercaptoethanol. The homogenate was strained through muslin and centrifuged at 15000 *g* for 15 min. The supernatant was brought to 40% saturation with $(NH_4)_2SO_4$ and centrifuged at 15000 *g* for 15 min. Resultant supernatant was adjusted to 65% saturation with $(NH_4)_2SO_4$ and ppt. formed after 30 min collected by centrifugation at 15000 *g* for 20 min. The pellet was resuspended in 5 ml KH_2PO_4 buffer, pH 8·5, 0·05 M containing 5 mM mercaptoethanol and loaded on to a Sephadex G-25 column. The eluate was used as the enzyme extract.

P. hispidus. Mycelium (100 g fr. wt) was ground with an equal wt of acid-washed sand in a chilled mortar and extracted (×2) with an equal vol. of KH_2PO_4 buffer, pH 7·5, 0·2 M containing 5 mM mercaptoethanol. Homogenate was strained through muslin and centrifuged at 15000 *g* for 15 min. 2% Protamine sulphate was slowly added to the stirred supernatant (1 ml:10 ml of extract). The ppt. was removed by centrifugation and the supernatant brought to 40% saturation with $(NH_4)_2SO_4$. The ppt. was removed by centrifugation and the supernatant re-adjusted to 60% saturation, the resultant precipitate being collected by centrifugation at 20000 *g* for 20 min. The pellet was suspended in a minimum of KH_2PO_4 buffer, pH 8·5, 0·05 M and applied to a Sephadex G-25 column. The eluate was used for the enzyme extract.

Assay for conversion of p-*coumarate to* p-*hydroxybenzoic acid* was conducted using the following reaction mixture: 2 μmol *p*-coumaric acid; 1 μmol NAD; 600 μmol KH_2PO_4 buffer, pH 8·5 containing 5 mM mercaptoethanol; 1 ml of enzyme extract in a total vol. of 1·2 ml. In the experiments with CoA and ATP the reaction mixture was supplemented with 0·5 μmol CoA and 10 μmol ATP. The reaction was run at 30° for 3 hr and terminated by the addition of 0·1 ml of 6N HCl. The solution was frozen and thawed once and inactivated protein removed by centrifugation. The solution was extracted into Et_2O and after removal of the ether the products taken up in EtOH and chromatographed on cellulose gel (Avicel, Brinkmann Instruments Ltd) TLC, solvent 2% formate. The strip corresponding to *p*-hydroxybenzoic acid was removed and eluted with EtOH acidified with a few drops of HOAc. The absorption at 255 nm was recorded and the amount of compound estimated from a molar extinction of 9600. The samples were measured against a blank from a reaction mixture containing no substrate.

Assay of p-*hydroxybenzaldehyde oxidase.* Reaction mixture consisted of 0·5 ml of enzyme extract; 1 μmol NAD; 350 μmol KH_2PO_4 buffer, pH 8·5 containing 5 mM mercaptoethanol; 2 μmol *p*-hydroxybenzaldehyde. Extraction and assay of *p*-hydroxybenzoic acid was as above. The reaction mixture was incubated at 30° for 2 hr.

Identification of reaction products; *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde and *p*-hydroxybenzyl alcohol were identified by their colour reaction with *p*-nitroaniline and R_f's in 2% formate and C_6H_6–HOAc–H_2O (10:7:3) which were very similar to those of authentic samples. Their UV spectra were also very similar to those of standards. Acetic acid was identified in the reaction mixture by GLC. Six separate reaction mixtures containing a total of 120 mg enzyme extract, 6 μmol NAD, 3·5 mmol KH_2PO_4, pH 8·5, containing 5 mM mercaptoethanol were incubated at 30° for 3 hr. The reaction was terminated with 6N HCl and extracted into 3 vols ether. The ether was reduced to dryness and the sample re-suspended in a minimum of ether. 1 μl was used in GLC in a Tracor 550 Gas Chromatogram using a Chromosorb 101 column (Mesh 60/80,) obtained from Alltech. The conditions were carrier (He) flow rate 60 ml/min at pressure 8 psi. Detector flow rate H_2 50 ml/min; at 12 psi; Air 1·2 CFH at 20 psi. Temperature program 70°–250° @ 15°/min. Flame ionisation detector. Protein was estimated by the method of Lowry [11].

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REFERENCES

1. El-Basyouni, S. Z., Chen, D., Ibrahim, R. K., Neish, A. C. and Towers, G. H. N., (1964) *Phytochemistry* **3,** 485.
2. Grisebach, H. and Vollmer, K. O. (1964) *Z. Naturforsch* **B19,** 781.
3. Zenk, M. H. (1966) in *Biosynthesis of Aromatic Compounds* (edited by Billek, G.), p. 45. Pergamon Press, Oxford.
4. Zenk, M. H. (1965) in *Proc. 2nd Meeting European Biochem. Soc.* Vienna (edited by Billek, G.), p. 45. Pergamon Press, Oxford.
5. Alibert, G. and Ranjeva, R. (1971) *FEBS Letters* **19,** 11.
6. Alibert, G., Ranjeva, R. and Boudet, A. (1972) *Biochim. Biophys. Acta* **279(2),** 282.
7. Kindl, H. and Ruis, H. (1971) *Z. Naturforsch* **B26,** 1379.
8. Toms, A. and Wood, J. M. (1970) *Biochemistry* **9,** 337.
9. Camm, E. L. and Towers, G. H. N. (1973) *Phytochemistry* **12,** 1575.
10. Vance, C. P., Tregunna, E. B., Nambudiri, A. M. D. and Towers, G. H. N. (1974) *Biochim. Biophys. Acta* **343,** 138.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.

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INHIBITORY ACTIVITY OF THE PHENOLIC GLUCOSIDE PSILOTIN AND ITS REVERSAL BY GIBBERELLIC ACID AND THIOLS

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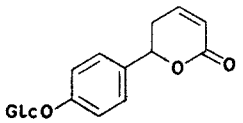
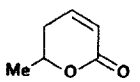
Key Word Index—*Psilotum nudum*; Psilotales; psilotin; unsaturated lactone; seed germination inhibitor; reversed by thio-compounds and gibberellin A_3.

Psilotin, 6-[4′-β-D-glucopyranosyloxyphenyl]-5,6-dihydro-2-oxo-2H-pyran (**1**), was first isolated by McInnes *et al.* [1] from *Psilotum nudum.* Subsequently, Tse *et al.* [2] isolated the compound from *Tmesipteris tannensis* but reported it to be absent from lycopods. McInnes *et al.* [1] called attention to the common structural relationship between psilotin (**1**), parasorbic acid (**2**) and massoilactone, namely the αβ-unsaturated δ-lactone ring, and with these comparisons we suggest inclusion of coumarin. Evidence for growth inhibitory activity in such structures [3] led to the suggestion that psilotin, too, might be biologically active. We can now report that psilotin is in fact an active inhibitor of seed germination and plant growth.



Psilotin (**1**)



Parasorbic acid (**2**)

RESULTS AND DISCUSSION

Seed germination was inhibited in all three species (Table 1). Seeds held for 4–6 days after maximum germination of controls failed to yield additional seedlings, hence were considered dead or dormant. Turnip seed germination and linear growth of seedlings required higher psilotin concentrations than corresponding inhibitions in the other species, and lettuce exhibited the greatest sensitivity. These differences were not reflected in fresh weight data. At a concentration of 10 mmol/liter, psilotin inhibited turnip and lettuce germination completely and limited onion to only 12%.

Table 1. Inhibitory activity of psilotin: ID_{50} values in seed germination and seedling growth by psilotin

Species	Germination	ID_{50} (mM) Seedling Growth (7 days) fresh wt	root length	shoot length
Turnip	5·2 ± 0·4	2·2 ± 0·2	1·6 ± 0·2	2·8 ± 0·2
Onion	1·6 ± 0·2	3·1 ± 0·2	0·8 ± 0·1	1·6 ± 0·2
Lettuce	1·6 ± 0·2	3·4 ± 0·2	0·3 ± 0·1	0·9 ± 0·1

Values were obtained by graphical interpolation of the concentration required for 50% inhibition. Inhibition data for germination was taken when control levels attained this maxima, 24, 48 and 72 hr respectively for turnip, onion and lettuce.

In general, root length was more sensitive to psilotin than other growth parameters in each species, and inhibi-