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ISOLATION AND PROPERTIES OF HYDROXYCINNAMATE: CoA LIGASE FROM *POLYPORUS HISPIDUS**

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Abstract—Hydroxycinnamate: CoA ligase was partially purified from the basidiomycete, *Polyporus hispidus*. The enzyme required ATP and CoA. Reduced activity was obtained with GTP. The same preparations catalyzed acetyl CoA formation. Light-grown cultures yielded preparations with an increased activity for hydroxycinnamic acids but not for acetate.

INTRODUCTION

CoA thiol esters of cinnamic acids have been postulated to be involved in many aspects of polyphenol metabolism in higher plants [1–3]. Recent evidence has shown them to be subtrates in the formation of flavonoids [4], chlorogenic acid [5] and cinnamoyl alcohols [6–8] and the ligases catalyzing the formation of CoA esters of cinnamic acids have been studied in a number of higher plants [9–13]. Perrin and Towers [14], have suggested that CoA esters of cinnamic acids are also involved in the biosynthesis of the styrylpyrones, hispidin [6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone] and *bis*-noryangonin [6-(4-hydroxystyryl)-4-hydroxy-2-pyrone] in the Basidiomycete, *Polyporus hispidus*.

In previous studies of styrylpyrone biosynthesis in *P. hispidus* we showed that light stimulated phenylalanine ammonia lyase, cinnamate-4-hydroxylase, *p*-coumaric acid hydroxylase and styrylpyrone biosynthesis [15, 16]. We have now demonstrated the presence of an enzyme in this fungus which can catalyze the esterification of CoA with hydroxycinnamic acids and which increases in activity in response to light.

RESULTS

Table 1 shows the partial purification of the hydroxycinnamate: CoA ligase from *P. hispidus*. No activity could be obtained with crude preparations either before or after treatment with Dowex 1. After $MnCl_2$ precipitation however, activity was detectable in the supernatant. Further fractionation with $(NH_4)_2SO_4$ removed endogenous inhibitors. The enzyme was very unstable at this stage, even in 5 mM mercaptoethanol, losing 80% of its activity in 24 hr and attempts to stabilize it have been unsuccessful to date. The 40–70% $(NH_4)_2SO_4$ fraction was passed through Sephadex G-25 and used immediately to determine substrate specificity, co-factor requirements, pH optimum and the effect of light.

The optimum pH for the activation of cinnamic acids was between 7·5 and 8·0. Below pH 7·0 and above 8·5 activity was greatly reduced. The substrate specificity of the enzyme towards five hydroxycinnamic acids and acetate was determined at pH 7. The highest activity was obtained with caffeic (4·75 nmol/min/mg protein) and *p*-coumaric acids (4·08). Ferulic and acetic acids were activated at approximately the same rate (2·68 and 2·20 nmol/min/mg protein respectively). No activation of cinnamic or 3,5-dimethoxycinnamic acid could be detected. The nucleotide requirement for the activation of *p*-coumaric acid showed that GTP (0·81 nmol ester/min/mg protein with 15 μmol of

* Part 3 in the series "Styrylpyrone biosynthesis in *Polyporus hispidus*". For Part 2 see Nambudiri, A. M. D., Vance, C. P. and Towers, G. H. N. (1974) *Biochem. Biophys. Acta.* **343**, 148.

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Table 1. Partial purification of hydroxycinnamate: CoA ligase from *Polyporus hispidus*

Purification step	Protein (mg)	Total activity (nmol/min)	Sp. act.* (nmol/min/mg protein)
Crude	264	0	0
Dowex (supernatant)	207	0	0
$MnCl_2$ (supernatant)	176	42·2	0·24
$(NH_4)_2SO_4$ fractionation 40–70%	20·8	44·7	2·15†

* *p*-Coumarate used as substrate in assay. Assay described in the Experimental. † 9-fold purification from previous step.

nucleotide) was 33% as effective as ATP (2·64) while no activity was detectable with CTP and UTP.

In the absence of CoA or ATP no activity was detectable. CoA at a concentration of 0·50 mM (3·88 nmol ester/min/mg protein) was most effective in activation of *p*-coumaric acid. This activity was twice that with CoA at 0·25 mM while if the coenzyme concentration was increased to 1 mM, the activity was decreased (2·82). A molar ratio of ATP to CoA of 30:1 appeared most effective in CoA ester formation.

The effect of light on the formation of CoA thiol esters of three cinnamic acids and acetate are shown in Table 2. Light significantly stimulates formation of *p*-coumaryl, caffeyl, and ferulyl CoA as compared to dark. In the dark there was slight formation of *p*-coumarate ligase activity but no detectable activation of ferulate or caffeate. There was little difference in the formation of acetyl CoA between light and dark grown cultures.

DISCUSSION

This is the first report of an enzyme capable of catalyzing the formation of CoA thiol esters of hydroxycinnamic acids in fungi. The fungal enzyme appears to be similar to the enzyme from higher plants in some respects. The results presented in Table 1 are in agreement with those of Gross and Zenk [12], Rhodes and Wooltorton [13], and Lindl *et al.* [11], with respect to the presence of an endogenous inhibitor which is removed during purification.

Table 2. The effect of light on hydroxycinnamate: CoA ligase of *Polyporus hispidus*.

Substrate	Sp. act.* (nmol/min/mg protein) Light grown*	Dark grown
p-Coumarate	1·51	0·29
Caffeate	1·84	0·00
Ferulate	1·00	0·00
Acetate	1·45	1·49

* Conditions of growth described in Experimental.

The substrate specificity of the enzyme from *P. hispidus* is similar to that of the enzyme from *Forsythia* [12] and swede root disks [13] (*Brassica napo-brassica*) showing highest activation with caffeic, *p*-coumaric and ferulic acids. It differs from the enzyme found in spinach beet leaves [10] which has highest activity towards cinnamic acid and is inactive with hydroxy-substituted cinnamic acids. The fungal enzyme does not activate cinnamic acid or 3,5-dimethoxycinnamic acid. GTP is about 33% as effective as ATP for activation of *p*-coumarate with the fungal enzyme, in contrast to the enzyme from swede root which uses only ATP for CoA formation [13].

Light stimulated levels of enzyme activity for hydroxycinnamic acids whereas the activity for the formation of acetyl CoA was relatively constant in light and dark grown cultures (Table 2). This would suggest that the enzyme responsible for the activation of hydroxycinnamic acids in *P. hispidus* is either a different enzyme than the one which activates acetate or that light alters the substrate specificity of a single enzyme making it more favorable for the activation of hydroxycinnamic acids. Recent reports by Gross and Zenk [12], and Ebel and Grisebach [17], indicate that there are separate enzymes for activation of acetate and hydroxycinnamic acids in plants. They also suggest that there may exist, in plants, several cinna-

mate: CoA ligases involved with different pathways of phenylpropanoid metabolism.

EXPERIMENTAL

Polyporus hispidus (UBC 513) was cultured as described in [16]. For enzyme extraction large numbers of cultures were maintained at 25°C in Psycrotherm incubators provided with "daylight" fluorescent tubes. The light-grown mycelium was derived from bottles exposed to light ½ hr/day, the dark-grown mycelium being totally unexposed to light. The mycelium was harvested at day 14.

Preparation of cell free extracts. These were obtained by grinding the mycelium with an equal wt of acid-washed sand in a chilled porcelain mortar and extracting (2×) with 0·2 M KH_2PO_4 buffer containing 5 mM mercaptoethanol pH 7·7 (2 ml/g of mycelium). Further purification was similar to that described by Lindl *et al.* [11]. Resultant slurry was filtered through cheesecloth, centrifuged at 15000 *g* for 15 min and the pellet discarded. The supernatant was stirred for 20 min with Dowex I-×8 (PO_4 form, equilibrated with 0·2 M KH_2PO_4 buffer pH 7·7, 1 g/12 ml supernatant), and filtered through glass wool. A soln of 1 M $MnCl_2$ was slowly added to the filtrate to give a final concn of 35 mM $MnCl_2$. After stirring for 20 min the preparation was centrifuged at 15000 *g* for 15 min and the pellet discarded. The supernatant was adjusted to 40% saturation with solid $(NH_4)_2SO_4$, the precipitate removed by centrifugation and the supernatant adjusted to 70% saturation with solid $(NH_4)_2SO_4$. The ppt formed in 45 min was centrifuged at 20000 *g* for 20 min and the pellet resuspended in 3 ml of 0·05 M KH_2PO_4 buffer containing 5 mM mercaptoethanol (pH 7·7). This soln was passed through Sephadex G-25 and the eluate used as the enzyme.

Assay for hydroxycinnamate: CoA ligase. The assay procedure used was similar to that described by Lindl *et al.* [11]. The reaction mixture for the hydroxamate assay included 50 μmol KH_2PO_4 buffer containing 5 mM mercaptoethanol pH 7·7, 0·5 μmol CoA, 10 μmol ATP, 3 μmol $MgSO_4$, 200 μmol KCl, 200 μmol hydroxylamine, 200 μmol KOH, 5 μmol substrate, 1 μmol DTT and 0·3–0·5 mg protein in a final vol. of 1·0 ml. The reaction mixture was incubated at 30° for 120–180 min and the formation of the hydroxamate was shown to proceed linearly with time over 4 hr. The reaction was stopped by adding 0·75 ml of an acidic soln of $FeCl_3$ [17]. Denatured protein was removed by centrifugation and the absorbance at 546 nm was measured against a blank containing either no substrate or no cofactors. The amounts of hydroxamic acid formed were calculated from the extinction coefficients determined by Gross and Zenk [19].

Identification of the product of the reaction. The product of the hydroxamate reaction with *p*-coumarate acid was assayed according to the procedure of Rhodes and Wooltorton [13]. The product was identical in characteristics to an authentic sample of *p*-coumaryl hydroxamate prepared by the method of Hahlbrock and Grisebach [9]. *p*-Coumaryl CoA thioester was isolated after a large scale reaction containing 10 μmol CoA, 100 μmol ATP, 60 μmol *p*-coumaric acid, 500 μmol KH_2PO_4 phosphate buffer containing 5 mM mercaptoethanol (pH 7·7), 30 μmol $MgSO_4$, 200 μmol KCl and 8 mg protein in a final vol. of 10 ml. The reaction mixture was incubated for 4 hr at 30° and stopped with 0·5 ml of 6 N HCl. Pptd protein was removed by centrifugation and supernatant evaporated to dryness at room temp. under red pres. Residue was dissolved in 2 ml of 50 mM HCOOH and the *p*-coumaryl CoA ester isolated by low temp. PC according to Gross and Zenk [19]. The ester had an R_f of 0·51 while the free acid ran close to the front (R_f 0·90). Elution from paper with 0·1 M KH_2PO_4 buffer, pH 7·5, gave a product with a UV max of 333 nm. Alkaline hydrolysis (0·5 N NaOH–60 min at room temp.) gave *p*-coumaric acid identified by UV and PC. Protein was measured according to the method of Lowry [20].

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REFERENCES

1. Grisebach, H. (1967) *Biosynthetic Patterns in Microorganisms and Higher Plants*, p. 2. Wiley, New York.
2. Zenk, M. H. and Gross, G. G. (1972) *Recent Adv. Phytochemistry* **4,** 87–106.
3. Vollmer, K. O., Reisner, H. J. and Grisebach, H. (1965). *Biochem. Biophys. Res. Commun.* **21,** 221–225.
4. Kreuzaler, F. and Hahlbrock, K. (1972) *FEBS Letters* **28,** 69–72.
5. Stockigt, J. and Zenk, M. H. (1974) *FEBS Letters* **41,** 131.
6. Mansell, R. L., Stockigt, J. and Zenk, M. H. (1972) *Z. Pflanzenphysiol.* **68,** 286.
7. Ebel, J. and Grisebach, H. (1973) *FEBS Letters* **30,** 141.
8. Rhodes, M. J. C. and Wooltorton, L. S. C. (1974) *Phytochemistry* **13,** 107.
9. Hahlbrock, K. and Grisebach, H. (1970) *FEBS Letters* **11,** 62.
10. Walton, E. and Butt, V. S. (1971) *Phytochemistry* **10,** 295.
11. Lindl, T., Kreuzaler, F., and Hahlbrock, K. (1973) *Biochem. Biophys. Acta.* **302,** 457.
12. Gross, G. G. and Zenk, M. H. (1974) *Eur. J. Biochem.* **42,** 453.
13. Rhodes, M. J. C. and Wooltorton, L. S. C. (1973) *Phytochemistry* **12,** 2381.
14. Perrin, P. W. and Towers, G. H. N. (1973) *Phytochemistry* **12,** 589.
15. Nambudiri, A. M. D., Vance, P. C. and Towers, G. H. N. (1973) *Biochem. J.* **134,** 891.
16. Vance, C. P., Tregunna, E. B., Nambudiri, A. M. D. and Towers, G. H. N. (1974) *Biochem. Biophys. Acta.* **343,** 138.
17. Ebel, J. and Grisebach, H. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354,** 1183.
18. Jones, M. E. and Lipmann, F. (1955) in *Methods in Enzymology* Vol. 1, (eds. Colowick, S. P. and Kaplan, N. O.) pp. 585–591. Academic Press, New York.
19. Gross, G. G. and Zenk, M. H. (1966) *Z. Naturforsch.* **21b,** 683.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.