FORMATION OF COENZYME A ESTERS OF CINNAMIC ACIDS WITH AN ENZYME PREPARATION FROM CELL SUSPENSION CULTURES OF PARSLEY

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The formation of coenzyme A thiol esters of cinnamic, *p*-coumaric, *p*-methoxy cinnamic, and ferulic acids was catalyzed by enzyme preparations from cell suspension cultures of leaf petioles from parsley (*Petroselinum hortense Hoffm.*). Of these acids, *p*-coumaric acid served as the most efficient substrate. Enzyme activity is markedly increased upon illumination with white light in a manner very similar to that in which the activities of a number of enzymes involved in flavone biosynthesis are stimulated by light. This strongly suggests that the formation of *p*-coumaroyl coenzyme A is part of this biosynthetic pathway.

1. Introduction

CoA thiol esters of cinnamic acids have long been postulated as precursors in the biosynthesis of flavonoids [1], lignin [2], and many other plant products. Very probably they are also involved in the degradation of cinnamic acids to benzoic acids [3] and other C_6-C_1-compounds [4] in higher plants.

In our earlier attempts to prove the formation of CoA esters of cinnamic acids with cell-free preparations from parsley and garbanzo bean (*Cicer arietinum L.*) we obtained only a very weak activation of *p*-coumaric acid. It was not possible to decide whether this activation was catalyzed by an enzyme specific for cinnamic acids or merely due to an unspecific action of another activating enzyme [5]. With the knowledge that the activity of various enzymes involved in the flavonoid pathway changes considerably during the growth of young parsley plants [6] and after illumination of cell suspension cultures from parsley [7], we have now been able to obtain enzyme preparations from cell suspension cultures of parsley which catalyze a strong formation of CoA esters of cinnamic acids.

2. Experimental

2.1. *Methods*

Cell suspension cultures from parsley leaf petioles were grown, illuminated with white light, and harvested as described in a previous communication [7]. For all of the experiments, except for the kinetic studies (fig. 1), the material was illuminated for 15 to 20 hr. 30 g of the cells were homogenized for 5 min in a mortar with 15 g of quartz sand and 15 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 20 µl of mercaptoethanol. After centrifugation for 15 min at 50,000 *g*, the protein was precipitated with solid ammonium sulfate (80% saturation), centrifuged off, and resuspended in 8 ml of the phosphate buffer. The solution was stirred for 30 min with 2 g of Dowex 1 (phosphate form, equilibrated with the buffer used), filtered through glass wool, and passed through a column of Sephadex G-25 (2 × 20) with 0.02 M potassium phosphate buffer (pH 7.5). The protein fraction was collected and used for enzyme assays.

A mixture containing 1 µmole of the acid tested, 1 µmole of CoA, 5 µmoles of ATP, 5 µmoles of $MgSO_4$, 400 µmoles of hydroxylamine, 8 µmoles of potassium phosphate, and 1 to 2 mg of protein in a total volume of 0.6 ml was incubated at 30° for 60

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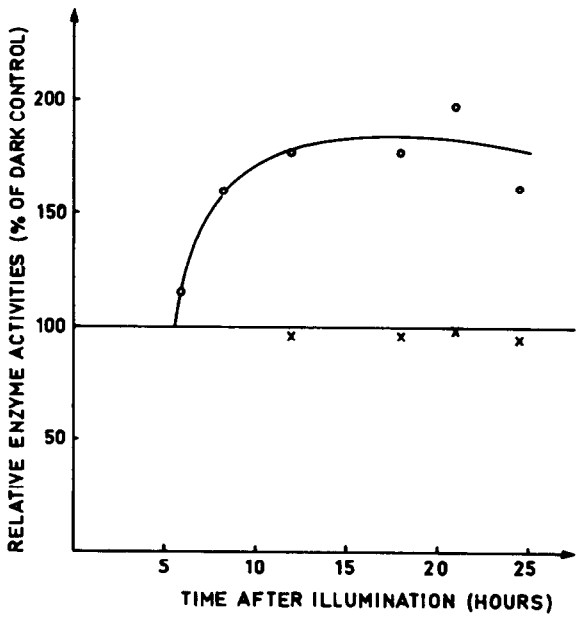

Fig. 1. Changes in relative activities of the enzymes activating *p*-coumaric acid (o) and acetic acid (x) after illumination with high intensities of white light (∿27 000 lux). Enzyme preparations were obtained from 3 g of cells as described under Methods, except that gel filtration was replaced by dialysis for 2 hr against 2 l of 0.02 M K phosphate buffer (pH 7.5).

min (pH 7.5). The reaction was stopped by adding 0.4 ml of an acidic solution of $FeCl_3$ [8] to the mixture and removing the denatured protein by centrifugation. The absorbance at 546 nm was measured against a blank containing no CoA. Nanomoles of hydroxamic acid formed were calculated using the extinction coefficients determined by Gross and Zenk [2].

For the isolation of ^{3}H-*p*-coumaroylhydroxamic acid from the incubation mixture, unlabelled material was added (sufficient to give a colour reaction with $FeCl_3$ after paper chromatography or electrophoresis), the solution was adjusted to pH 4–5 by addition of 6 N HCl, and then twice the volume of acetone was added. This mixture was centrifuged, and the supernatant was evaporated to dryness. Labelled material was extracted from the residue with acetone and used for paper chromatography and high voltage electrophoresis.

2.2. *Materials*

^{3}H-Labelled *p*-coumaric acid was prepared from 3,5-^{3}H-*p*-hydroxybenzaldehyde (1.7 μCi/μmole) as described by Bennet and Kirby [9]. *p*-Coumaroylhydroxamic acid was synthesized by adding 10 ml of a freshly prepared mixture of equal amounts of 5% hydroxylamine hydrochloride and 8% potassium hydroxide in methanol to 10 mg of *p*-coumaric acid methyl ester. After 45 min at room temperature, the solution was adjusted to pH 6 with conc. hydrochloric acid, filtered, and evaporated to dryness. The residue was extracted with 2 ml of acetone. Most of the solvent was then removed, and the remaining oily mixture was used for chromatographic and electrophoretic studies.

3. Results and discussion

Table 1 summarizes the results obtained with two different enzyme preparations. In both cases, *p*-coumaric acid was the most effective substrate of the aromatic acids tested. Caffeic acid, another possible substrate, forms a strongly coloured complex with ferric ions and can therefore not be tested by this method. Under the assay conditions used, the amount of hydroxamic acid formed is directly proportional to the amount of protein (up to approx. 2 mg) and to the time of incubation over a period of at least 120 min. No reaction was observed when

Table 1
Activation of various acids with enzyme preparations from cell suspension cultures of parsley.

Acid	Hydroxamic acid formed (nmoles)	
	Expt. 1	Expt. 2
Cinnamic	54	32
p-Coumaric	133	80
p-Methoxycinnamic	62	40
Ferulic	103	60
Acetic	–	121
Malonic	–	29

Enzyme activity was assayed with the hydroxamic acid test [8].

Table 2
Identity of the reaction product in the activation test with *p*-coumaroyl hydroxamic acid.

Compound	R_f (paper chromatography)	Electrophoretic migration distance
p-Coumaric acid	0.90	+6.9 cm
^{3}H-*p*-Coumaric acid	0.90	+6.5 cm
p-Coumaroyl hydroxamic acid	0.75	−0.9 cm
^{3}H-Reaction	0.74	−0.8 cm

Descending paper chromatography on Whatman 3 MM paper was carried out with the solvent system butanol-acetic-H_2O (20:1:4). High voltage paper electrophoresis was carried out on Whatman No. 1 paper in pyridine-acetic acid-H_2O (10:10:1560, pH 4.7) for 2 hr at 50 V/cm.

either CoA, ATP or $MgSO_4$ was omitted from the incubation mixture, or when heat-inactivated enzyme was used. Within 1 hr, up to 15% of the coumaric acid was converted to the hydroxamate. It should be noted that neither aliphatic nor cinnamic acids were activated by crude extracts from the cells unless compounds of low molecular weight were removed by ammonium sulfate precipitation of the protein and treatment with Dowex 1. Additional purification of the protein by gel filtration resulted in a further increase in enzyme activity.

In earlier experiments with extracts from leaves of parsley or garbanzo bean [5] much higher activation rates were found for acetate and malonate than for cinnamic acids. In contrast, with enzyme preparations from cell suspension cultures of parsley the formation of CoA esters of all the acids listed in table 1, expt. 2, was approximately of the same order of magnitude. The identity of the reaction product in the hydroxamic acid assay with *p*-coumaric acid hydroxamate was confirmed by paper chromatography and paper electrophoresis of ^{3}H-labelled *p*-coumaroyl-hydroxamic acid (table 2). In both cases, the radioactivity migrated with the reference compound which was detected by its colour reaction with $FeCl_3$.

An important feature of the *p*-coumaric acid activating enzyme is the stimulation of its activity by light. Whereas the formation of acetyl-CoA is not influenced by light at all, enzyme activity for the activation of *p*-coumaric acid is markedly increased after a lag of about 5 hr after illumination (fig. 1). Since a great number of enzymes involved in the biosynthesis of flavone glycosides as well as the accumulation of these compounds in cell cultures from parsley are also stimulated light with the same lag period of 4–6 hr [7, 10], it is concluded from the results presented here that the fomarion of *p*-coumaroyl-CoA is catalyzed by a specific enzyme directly related to the biosynthesis of flavonoids.

We have also been able to demonstrate the activation of *p*-coumaric acid with cell-free preparations from young parsley leaves and from cell suspension cultures of soybean (*Soja hispida*). Further studies on the purification and properties of the enzyme are in progress.

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References

[1] H.Grisebach, Biosynthetic Patterns in Microorganisms and Higher Plants (Wiley, New York, 1967) p. 2.
[2] G.G.Gross and M.H.Zenk, Z.Naturforsch. 21b (1966) 683.
[3] K.O.Vollmer, H.J.Reisener and H.Grisebach, Biochem. Biophys. Res. Commun. 21 (1965) 221.
[4] M.H.Zenk, Proc. 2nd Meeting FEBS 1965, Vol. 3 (Pergamon Press, Oxford 1966) p. 45.
[5] K.Hahlbrock, Doctoral thesis, Freiburg i. Br. 1965; H.Grisebach, W.Barz, K.Hahlbrock, S.Kellner and L. Patschke, Proc. 2nd Meeting FEBS 1965, Vol. 3 (Pergamon Press, Oxford, 1966) p. 25.
[6] K.Hahlbrock, A.Sutter, E.Wellmann, R.Ortmann, and H.Grisebach, Phytochemistry, in press.
[7] K.Hahlbrock and E.Wellmann, Planta 94 (1970) 236.
[8] E.R.Stadtman, in: Methods in Enzymology, Vol. 3, eds. S.P.Colowick and N.O.Kaplan (Academic Press, New York 1957) pp. 228–231.
[9] D.J.Bennet and G.W.Kirby, J. Chem. Soc. (1968) 422.
[10] K.Hahlbrock, A.Sutter and H.Grisebach, unpublished results.