REDUCTION OF CINNAMIC ACIDS TO CINNAMYL ALCOHOLS WITH AN ENZYME PREPARATION FROM CELL SUSPENSION CULTURES OF SOYBEAN (*GLYCINE MAX*)

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1. Introduction

Tracer studies and model experiments have amply demonstrated that cinnamyl alcohols are the primary building stones of lignin [1]. Evidence for the reduction of ferulic acid to coniferylalcohol via coniferylaldehyde has been obtained from tracer experiments [1, 2]. Since this reduction is an endergonic process, it is to be expected that the enzymatic reaction requires the activation of the carboxyl group. However, attempts to demonstrate a direct reduction of cinnamoly CoA esters *in vitro* with plant extracts have so far failed [6]*. Recently, the formation of coenzyme A thiol esters of cinnamic acids with enzyme preparations from plants [3] or cell suspension cultures of plants [4, 5] has been shown. In this communication we report on an enzyme preparation from cell suspension cultures of soybean catalyzing the reduction of feruloyl CoA (I) to coniferylalcohol (III) and p-coumaroyl CoA (II) to p-coumarylalcohol (IV).

2. Materials and methods

2.1. *Materials*

Biochemicals were purchased from Boehringer, Mannheim. Coniferylalcohol was obtained from Fa. C. Roth, Karlsruhe. p-Coumarylalcohol was prepared from acetyl-p-methylcoumarate [12]. p-[2-^{14}C]Coumaric acid (3.2 mCi/mmole) and [2-^{14}C]ferulic acid (5.7 mCi/mmole) were synthesized by the reaction of [2-^{14}C]malonic acid (Radiochemical Centre, Amersham) with p-hydroxybenzaldehyde and vanillin, respectively, according to published procedures [13].

p-Coumaroyl CoA and p-[2-^{14}C]coumaroyl CoA were a gift from F. Kreuzaler, Freiburg.

The 2,4-dinitrophenylether of coniferylalcohol was prepared as described by Freudenberg and Lehmann [9].

2.2. *Separation and analysis*

Descending paper chromatography was carried out on Whatman 3 MM with solvent system **1**, n-butanol saturated with 2% aqueous NH_3 [14], and on Schleicher and Schull 2043b with **2**, xylene–dimethylformamide (9:2, v/v) [9]. Thin-layer chromatography was performed on silica gel with **3**, benzene–dioxane–acetic acid (90:25:4, v/v); **4**, benzene–ethanol (92:8, v/v); **5**, benzene–acetic acid (8:2, v/v); **6**, toluene–ethylformate–formic acid (5:4:1, v/v) and on cellulose (Macherey and Nagel) with **7**, 2% acetic acid.

2.3. *Soybean cells*

Cell suspension cultures of *Glycine max* were grown as described previously [5]. Cells were harvested when the conductivity of the culture medium indicated maximum activity of phenylalanine ammonia-lyase [7] and p-coumarate:CoA ligase [5].

2.4. *Enzyme preparation*

All steps were carried out at 4°. Two g of wet cells were ground for 3 min in a chilled mortar with 1g of

* After completion of this manuscript a preliminary communication by Zenk et al. appeared in which the reduction of ferulic acid to coniferylalcohol with an enzyme preparation from cambial tissue of *Salix alba* was reported [16].

quartz sand and 2 ml of 0.2 M Tris-HCl (pH 7.5) containing 30 mM mercaptoethanol. After centrifugation for 10 min at 10 000 g ml of the supernatant was stirred for 10 min with 0.3 g of Dowex 1 X 2 (Cl^- form, equilibrated with 0.2 M Tris-HCl, pH 7.5) and filtered through glass wool. Final protein conc. was between 2.5 and 4 mg/ml.

2.5. *Enzyme assay*

The incubation mixture containing 100 nmoles of [2-^{14}C]ferulic acid (10^5 cpm), 2 μmoles of ATP, 80 nmoles of CoASH, 1 μmole of $MgCl_2$, 200 nmoles of NADPH, 30 μmoles of Tris-HCl (pH 7.5) and 200–300 μg of protein in a total volume of 150 μl was incubated at 30° for 20 min. At the end of this period 20 μl of a solution of 30 μg of coniferylalcohol in ethylene glycol monomethylether was added to the incubation mixture and the solution was then applied as a 4 cm long band to Whatman 3 MM. The chromatogram was developed with solvent system 1. The zone of coniferylalcohol (R_f = 0.8) was detected under UV light (254 nm), cut out and counted in a dioxane cocktail with a liquid scintillation spectrometer.

3. Results and discussion

Cell suspension cultures of soybean (*Glycine max*) form a lignin-like substance during their growth [8]. It had been shown previously that several enzymes related to phenylpropane metabolism, e.g. phenylalanine ammonia-lyase (EC 4.3.1.5) and p-coumarate:CoA ligase, show high activities only during a short period before the stationary phase of the cell cultures is reached [5]. Since it was expected that other enzymes of this pathway would also be most active during the same growth phase, the search for a reductase was started with extracts prepared from cells of this phase.

When crude extracts or extracts treated with Dowex 1 X 2 were incubated with [2-^{14}C]ferulic acid, ATP, CoASH, Mg^{2+} and NADPH only one radioactive product was formed, which proved to be coniferylalcohol (III). The enzymatic product was identified as III by chromatographic comparison with an authentic reference sample in solvent systems 1 and 3–7. After dilution with unlabeled III the radioactive product was further characterized by its 2,4-dinitrophenylether [9], which in solvent system 2 gave a radioactive peak with the same R_f-value as a reference sample [9].

Table 1

Reduction of [2-^{14}C]ferulic acid to coniferylalcohol with enzyme preparations from cell suspension cultures of soybean. Incubations were carried out in the standard assay.

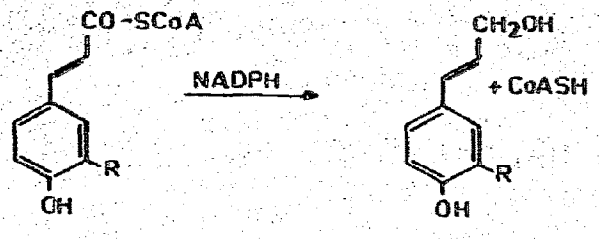
Incubation	Coniferyl-alcohol (cpm)	Relative activity (%)
Complete plus crude extract	4220	46
Complete plus Dowex-treated extract	9280	100
Complete plus heat-inactivated Dowex-treated extract	0	0
–ATP	75	0.8
–CoASH	115	1
–Mg^{2+}	4610	50
–Mg^{2+} + 3 mM EDTA	790	9
–NADPH	730	8
–NADPH + NADH	1340	14

After incubation of 2 μmoles of ferulic acid with 20 ml of Dowex treated protein extract and purification of the product by chromatography on Sephadex-LH-20 with methanol as solvent, sufficient product was obtained to take a UV spectrum. This proved to be identical with that of III (λ_{max} = 264 nm with inflection at 295 nm, in methanol) [15].

With free ferulic acid as substrate the reduction requires ATP, Mg^{2+}, CoASH and NADPH (table 1). NADH has about 10% of the activity of NADPH. Under the assay conditions used the amount of coniferylalcohol formed is linear with protein conc. (up to approx. 2 mg/ml) and time (at least 20 min).

p-Coumaric acid can also serve as substrate for the enzymatic reduction. When 100 nmoles (5.5×10^5 cpm) of p-[2-^{14}C]coumaric acid were used with a protein extract not treated with Dowex, a radioactive peak (1650 cpm) was detected at the position of p-coumarylalcohol (R_f = 0.85). NADPH was the only cofactor required for the formation of this product (2750 cpm), when 25 nmoles (10^5 cpm) of p-[2-^{14}C]coumaroyl CoA were used as substrate.

From the results presented it can be concluded that soybean cell cultures contain an enzyme system capable of catalyzing the reduction of cinnamoyl



I. R = OMe
II. R = H

III. R = OMe
IV. R = H

Fig. 1

CoA esters to the corresponding alcohols with NADPH as reductant (fig. 1). Purification of this enzyme is in progress.

Whether the reduction proceeds via the free aldehyde as suggested by tracer experiments [2] or via a bound aldehyde as in the case of 3-hydroxy-3-methylglutaryl coenzyme A reductase [10, 11] will be the subject of further investigation.

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