

REDUCTION OF COENZYME A THIOESTERS OF CINNAMIC ACIDS WITH AN ENZYME PREPARATION FROM LIGNIFYING TISSUE OF *FORSYTHIA*

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Received 24 March 1975

1. Introduction

It is well established that the lignin macromolecule is built up by the condensation of differently ring-substituted cinnamyl alcohols [1]. By tracer experiments it has been shown that these alcohols must be formed by the reduction of the corresponding cinnamic acids [1,2]. Such a reaction is an endergonic process requiring activation of the carboxyl group of the acid, and cinnamoyl-CoA esters have been postulated as these intermediates [3,4]. In fact, it was demonstrated by in vitro experiments, with extracts from higher plants, that the reduction of *p*-coumarate and ferulate was dependent on the presence of CoA [5–8]. Furthermore, authentic *p*-coumaroyl-CoA and feruloyl-CoA were found to be reduced in the presence of NADPH by extracts from soybean cell cultures [6] or from lignifying tissue of *Forsythia* [7,9]. In the present paper we describe some properties of the enzyme from *Forsythia* which catalyzes the reduction of cinnamoyl-CoA esters.

2. Experimental

2.1. Materials

The CoA esters of cinnamate, *p*-coumarate, *p*-methoxycinnamate, caffeate, ferulate, 3,4-dimethoxycinnamate and protocatechuate were prepared using acyl-CoA synthetase (EC 6.2.1.2) from beef liver mitochondria [3]. Purification of the reaction products was achieved by chromatography at 4°C on DEAE-cellulose columns using a linear gradient of sodium formate [10]. The CoA ester containing fractions were pooled, desalted [10] and lyophilized. Recoveries of the purified

products, based on the initial amount of CoA, were between 50 and 75%. Malonyl-CoA, chemically synthesized 5-hydroxyferuloyl-CoA and sinapoyl-CoA [11], and [methyl-³H]ferulic acid were gifts of Dr J. Stöckigt of this laboratory. Acetyl-CoA, benzoyl-CoA and other biochemicals were from Boehringer, Mannheim. Specifically labelled A and B forms of [4(n)-³H]NADPH were prepared as described previously [12].

2.2. Enzyme preparation

Young lignifying stems of *Forsythia suspensa* were freed of extracambial tissue, cut into small segments, frozen in liquid nitrogen and powdered in an ultracentrifugal mill (Retsch KG., Haan). All following steps were carried out at 0–4°C; buffer solutions were routinely supplemented with 20 mM 2-mercaptoethanol. 50 g of frozen powder were mixed with 50 g (wet weight) of prewashed polyclar AT and extracted for 1 hr with 150 ml of 0.1 M borate buffer, pH 7.8. The suspension was squeezed through muslin. After centrifugation of the filtrate, the supernatant was stirred for 15 min with 5 g of Dowex 1-X4 (borate) and filtered through glass-wool. The filtrate was fractionated with solid ammonium sulfate. The pellet obtained at 40–65% saturation was resuspended in 5 ml borate buffer, clarified by centrifugation and passed through a Sephadex G-200 column (2.5 × 70 cm). The most active fractions were combined, supplemented with bovine serum albumine (2 mg/ml) and stored at 0°C.

2.3. Assay procedures

For the measurement of cinnamoyl-CoA reductase activity, a photometrical assay was employed in which the decrease in A_{345} was followed, with feruloyl-CoA

as standard substrate [9]. Reaction mixtures, containing 50 μmol Tris-HCl, pH 7.8, 30 nmol feruloyl-CoA and 50–100 μg of enzyme in a total vol of 0.5 ml, were pipetted into semi-micro cuvettes and the blank reaction was determined. Reduction was then initiated by the addition of 150 nmol NADPH. To avoid errors caused by the spectral properties of accumulating aldehyde, cinnamyl alcohol dehydrogenase [12] was added to the assays when this enzyme was not present in the enzyme preparation. Therefore, the observed decrease in A_{345} was due to the combined disappearance of the thioester linkage and the oxidation of 2 mol of NADPH per mol of feruloyl-CoA reduced. Hence, the sum of the ϵ values of feruloyl-CoA (19 $\text{cm}^2/\mu\text{mol}$ [3]) plus 2 NADPH ($2 \times 6 \text{ cm}^2/\mu\text{mol}$) was used for the calculation of reaction rates. Under these conditions, the reduction of 1 nmol of feruloyl-CoA is equivalent to a decrease in A_{345} of 0.062. Reaction rates with other cinnamoyl-CoA substrates were assayed analogously, using the appropriate λ_{max} and ϵ values [3,11].

Since the spectral data of the CoA esters change with varied pH values, the pH optimum of the reaction was determined with [methyl- ^3H] feruloyl-CoA as substrate. Assay mixtures were incubated for 30 min at 30°C; the amount of reduced products was determined as described previously [13].

Protein concentrations were measured photometrically [14].

3. Results

3.1. Preparation and stability of cinnamoyl-CoA reductase

No enzymatic activity was observed during the initial purification steps. After ammonium sulfate fractionation, slight activity was found which was further increased by gel chromatography, thus indicating the successive removal of inhibitory material. The reductase proved to be very unstable. Inactivation was retarded by thiol compounds and the addition of serum albumine to the partially purified enzyme. Under these conditions, gradually decreasing enzymatic activity could be maintained for a maximum of 3–4 days. From gel filtration experiments, a mol. wt of approx. 40 000 was calculated for the reductase.

3.2. Characteristics and properties

As shown in table 1, the enzyme requires feruloyl-

Table 1
Cofactor requirement for cinnamoyl-CoA reductase

System	Reaction rate (nmol/min)	Rate relative to the complete system %
Complete	0.24	100
–feruloyl-CoA	0	0
–NADPH	0.0016	0.7
–NADPH, + NADH	0.0016	0.7
–enzyme preparation	0	0
with heat-denatured enzyme	0	0

Assay conditions are as described in the experimental section. The data have been corrected for blank reactions due to non-enzymatic transacylation. For further details, see text.

CoA and NADPH as substrates. NADH could not substitute for NADPH. No requirement for further cofactors was observed. Neither the addition of divalent cations (Mg^{2+} , Mn^{2+} , Zn^{2+} , Fe^{2+}) nor the presence of EDTA had appreciable effects.

The initial velocity in the optical assay was proportional to enzyme concentration up to at least 100 μg (0.1 mU) of protein. No reaction occurred in the absence of enzyme solution. However, when heat-denatured enzyme was assayed, a slight decrease in A_{345} was observed. This fact led to the assumption that the earlier reported 'thiol esterase activity' [9] was due, at least in part, to nonenzymatic conversion of feruloyl-CoA; most likely caused by transacylation of ferulate to the 2-mercaptoethanol included in the enzyme solution (cf. [15]).

A closer analysis showed that this nonenzymatic reaction was dependent on the nature and concentration of the added thiol. With [methyl- ^3H] feruloyl-CoA and dithiothreitol, evidence for the formation of feruloyl dithiothreitol was obtained. The use of 2-mercaptoethanol, however, resulted in a practicable compromise between the formation of this artefact and the necessity of protecting the enzyme by thiols.

The reduction of feruloyl-CoA was found to proceed optimally in potassium phosphate or Tris-HCl buffer at pH values between 7.4 and 7.8. Determination of the effect of varied substrate concentration revealed optima at 0.06 mM concentration for feruloyl-CoA (K_m 22 μM) and 0.25 mM for NADPH (K_m 45 μM).

Table 2
Specificity of cinnamoyl-CoA reductase towards various cinnamoyl-CoA esters

Substrate	Relative activity %
Cinnamoyl-CoA	10
<i>p</i> -Coumaroyl-CoA	20
<i>p</i> -Methoxycinnamoyl-CoA	25
Caffeoyl-CoA	10
Feruloyl-CoA	100
3,4-Dimethoxycinnamoyl-CoA	40
5-Hydroxyferuloyl-CoA	20
Sinapoyl-CoA	20

Determinations were carried out under the conditions of the standard assay, except for the varied wavelengths in the photometric assay according to the different λ_{\max} values of the CoA esters. The activity with feruloyl-CoA (0.34 nmol/min) was taken as 100%.

Higher amounts of both substrates caused a drastic inhibition of the enzymatic reaction.

Table 2 shows the specificity of the enzyme towards various cinnamoyl-CoA esters. Feruloyl-CoA was converted most readily. No reaction occurred when the CoA esters of benzoate, protocatechuate and malonate were assayed at 340 nm. With acetyl-CoA, an oxidation of NADPH equivalent to 7% of the reaction rate as determined with feruloyl-CoA was observed.

3.3. Identification of reduced products

Most enzyme preparations contained varying amounts of cinnamyl alcohol dehydrogenase [12] which caused the formation of coniferyl alcohol as the final product. With [methyl- ^3H] feruloyl-CoA as substrate, labelled coniferyl alcohol was extracted from the reaction mixture and identified chromatographically. On several occasions, however, we were able to obtain enzyme preparations with no detectable contaminating alcohol dehydrogenase. After incubation of radioactive feruloyl-CoA with these preparations, labelled coniferyl aldehyde was isolated as the 2,4-dinitrophenylhydrazine derivative. After chromatography in two solvents [9], only one radioactive compound was observed and in both cases it was coincident with authentic material.

3.4. Stereospecificity of H-transfer

Stereospecificity in the reduction of feruloyl-CoA was determined by testing specifically labelled A and B forms of [4(n)- ^3H] NADPH. Reduction products were extracted with ether, separated by chromatography, and radioactivity of the compounds determined by liquid scintillation counting. With A-NADPH, only negligible radioactivity (71 cpm) was found in the coniferyl aldehyde; with the B-form, radioactivity of the aldehyde (1471 cpm) was 20.7 times higher. Thus, cinnamoyl-CoA reductase must belong to the B-group of NAD(P)-specific dehydrogenases.

4. Discussion

This communication reports the partial purification and some characteristics of an enzyme from lignifying tissue of *Forsythia* which catalyzes the reduction of cinnamoyl-CoA esters, preferably of feruloyl-CoA. In contrast to this, CoA esters of aliphatic or benzoic acids were converted scarcely or not at all. Furthermore, the enzyme is absolutely dependent on NADPH as cosubstrate. Based on these results, we propose the systematic name cinnamoyl-CoA: NADP oxidoreductase, or, according to the best substrate, feruloyl-CoA: NADP oxidoreductase (cf. [9]).

With feruloyl-CoA as substrate, and in the absence of cinnamyl alcohol dehydrogenase [12], the formation of coniferyl aldehyde was established unequivocally. This supports the conclusions reached in earlier in vivo [2] and in vitro experiments [5,7,9], and also rules out the possibility that the reaction proceeds directly to the alcohol as discussed by Ebel and Grisebach [6]. The latter is known for 3-hydroxy-3-methylglutaryl-CoA reductase, with an hemithioacetal participating as intermediate [16].

The enzyme described here was shown to be specific for B- ^3H -NADPH. The alcohol dehydrogenase further reducing the formed aldehyde has been shown to be of class A [12]. This result does not support the generalization of Davies et al. [17] that consecutive NAD(P) dependent dehydrogenases in a metabolic sequence should have the same stereospecificity. This diversity was also found for the reduction of aromatic acids in *Neurospora crassa* where the acid reductase belongs to class B [13] and the subsequent alcohol dehydrogenase is of class A (Gross, unpublished).

In addition to the enzyme described here, a hydroxycinnamate: CoA ligase [18,19] and a cinnamyl alcohol dehydrogenase [12] have also been isolated from this tissue. These enzymes also show an expressed specificity for phenylpropanoid substrates, and it is reasonable to assume that these three enzymes will catalyze the metabolic sequence leading from cinnamic acids to the corresponding alcohols, the immediate precursors of lignin.

Acknowledgements

The support and valuable criticism of Professor Dr M. H. Zenk and the skillful technical assistance of Mrs W. Conrad are gratefully acknowledged. This work was supported by a grant from the Deutsche Forschungsgemeinschaft to Professor Zenk.

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