THREE NOVEL ENZYMES INVOLVED IN THE REDUCTION OF FERULIC ACID TO CONIFERYL ALCOHOL IN HIGHER PLANTS: FERULATE:CoA LIGASE, FERULOYL-CoA REDUCTASE AND CONIFERYL ALCOHOL OXIDOREDUCTASE

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Received 1 March 1973

1. Introduction

Using a cell-free system from cambial tissue of *Salix alba* the reduction of ferulate to coniferyl alcohol has been shown for the first time, unequivocally, to occur in higher plants [1]. This conversion is dependent on ATP, CoA and reduced pyridine nucleotides. A reaction sequence has been postulated on the basis of these experiments involving the intermediate formation of feruloyl-CoA and coniferyl aldehyde which is in accordance with previous assumptions for this mechanism in lignin biosynthesis as recently reviewed [2].

In the present paper, a cell-free preparation of a lignifying higher plant which reduces ferulic acid to coniferyl alcohol is described. The overall reaction is composed of three individual enzymatic steps. Proof is presented that ferulate is first activated by a cinnamate: CoA ligase to feruloyl-CoA which is in turn reduced by NADPH to coniferyl aldehyde. This compound is further reduced to coniferyl alcohol, with NADPH again participating as the reductant.

2. Materials and methods

Radioactive ferulic acid-(O-CH_2T) (34 μCi/μmole) and coniferyl aldehyde of the same specific activity were prepared [3]. Feruloyl-CoA was prepared and purified as previously described [4].

As an enzyme source young stems, freed of extracambial tissue, of phytotron grown *Forsythia sp.* were used. The tissue was frozen in liquid nitrogen and powdered. To the powder was added polyclar AT (w/w) and the mixture subsequently extracted with 0.1 M borate buffer pH 7.8 supplemented with 10^{-2} M 2-mercaptoethanol. After centrifugation the supernatant was fractionated with ammonium sulfate and the fraction between 35 and 72% was resuspended in the same buffer as above. This solution was treated with an anion exchanger and used as a crude enzyme source. For the detection of the feruloyl-CoA reductase, the above enzyme solution was further fractionated on a Sephadex G-200 column.

Ferulate:CoA ligase was assayed by an optical method [4] using an ϵ_{345} of 19×10^6 $cm^2 \cdot mole^{-1}$. Spectrophotometric and tracer assays were employed for the detection of feruloyl-CoA reductase and coniferyl alcohol oxidoreductase.

Coniferyl aldehyde was trapped as an intermediate of the overall reaction using phenylhydrazine. The aldehyde was liberated from the phenylhydrazone by the addition of excess α-ketoglutarate, extracted into chloroform, and the 2,4-dinitrophenylhydrazone derivative prepared. This derivative was chromatographed on thin-layer plates in (I) benzene:ethyl acetate, 95:5 and (II) benzene:glacial acetic acid:H_2O, 6:7:3 (organic phase), and the plates scanned for radioactivity.

3. Results

3.1. *Ferulate: CoA ligase*

Using a crude enzyme preparation from tissue of *Forsythia* our results which had been obtained with

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North-Holland Publishing Company – Amsterdam

Table 1
Cofactor requirement for ferulate: CoA ligase.

System	Feruloyl-CoA formed (nmole·min^{-1})	Rate relative to the complete system (%)
Complete	1.7	100
minus dithiothreitol	1.5	88
minus Mg^{2+}	0.7	41
minus ferulate	0	0
minus CoA-SH	0	0
minus ATP	0	0
with heat-denatured enzyme	0	0

The reaction mixture contained in a final volume of 0.5 ml 25 μmoles Tris-HCl, pH 7.8, 5 μmoles $MgCl_2$, 1 μmole dithiothreitol, 0.5 μmoles ferulate, 0.25 μmoles CoA-SH, 1 μmole ATP and 0.17 mg protein. The reaction was followed spectrophotometrically at 345 nm (temperature 30°).

Salix [1] could be confirmed. The *Forsythia* enzyme had, however, the advantage that the overall reaction showed an absolute requirement for CoA as well as ATP and reduced pyridine nucleotide in the reduction of ferulate to coniferyl alcohol.

The reduction of ferulate to the corresponding alcohol is an endergonic process and most likely requires activation of the carboxyl group. The involvement of CoA and ATP strongly suggests the activation of ferulic acid via a CoA-thiolester. Since the spectra of CoA-thiolesters of cinnamic acids show a characteristic peak in the near UV at wavelengths between 311 and 363 nm a sensitive optical test can be employed to detect cinnamate:CoA ligase [4]. This test has been used to detect the ligase in our cell-free preparations. As shown in table 1, the activation reaction is completely dependent on ferulate, CoA and ATP. Mg^{2+} and a reduced thiol are necessary for full enzymatic activity. In order to prove the formation of feruloyl-CoA during this reaction, three ml of the reaction mixture (cf. table 1) were allowed to react for a period of 120 min. The incubation was worked up for cinnamoyl-CoA derivatives according to the general method given previously [4]. The purified product gave the absorption spectrum shown in fig. 1, with the characteristic absorption maximum at 345 nm. This spectrum is identical with that obtained from authentic feruloyl-CoA [4].

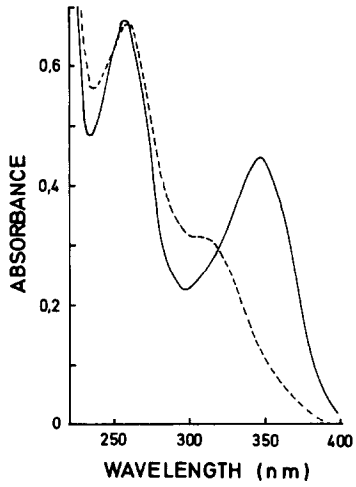

Fig. 1. UV-spectrum of feruloyl-CoA synthesized under the catalysis of ferulate:CoA ligase from *Forsythia*. Solid line: feruloyl-CoA; dashed line: spectrum after hydrolysis in 0.1 N NaOH. Both spectra recorded in 0.1 M phosphate buffer, pH 7.0.

The product showed the delayed response with sodium nitroprusside yielding a yellow color, characteristic of the thioester linkage of feruloyl-CoA. Furthermore, the absorption peak at 345 nm disappeared upon alkaline hydrolysis (fig. 1) yielding free ferulic acid as one of the reaction products. Treatment of the CoA-ester with 1 M NH_2OH at pH 7.0 led also to the disappearance of the absorption peak at 345 nm. In this case, feruloyl hydroxamate was found to be the reaction product. On the basis of these experiments we are able to conclude that the intermediate which accumulates under these reaction conditions and in the absence of reduced pyridine nucleotides, is feruloyl-CoA. The stoichiometry of the ferulate:CoA ligase reaction will be reported elsewhere.

3.2. *Feruloyl-CoA reductase*

Since feruloyl-CoA has been proven to accumulate, in the absence of reduced pyridine nucleotides, the possibility existed that either feruloyl-CoA was reduced by a specific feruloyl-CoA reductase to coniferyl aldehyde or that it was reduced directly to coniferyl alcohol. The *in vivo* findings of Higuchi and Brown [5] would suggest the first possibility, and in our earlier *in vitro* experiments [1], there was evidence that coniferyl aldehyde occurs as an intermediate. In order to test this hypothesis, feruloyl-CoA was

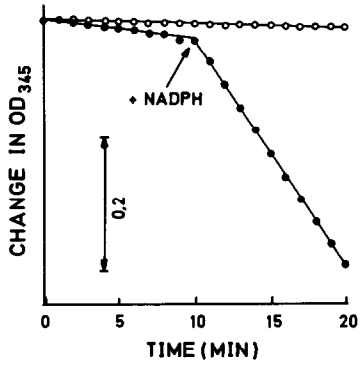

Fig. 2. Photometric assay for the reduction of feruloyl-CoA by feruloyl-CoA reductase from *Forsythia*. The complete reaction mixture contained in a final volume of 0.5 ml 50 μmoles Tris-HCl, pH 7.6, 5 μmoles $MgCl_2$, 1 μmole dithiothreitol, 20 nmoles feruloyl-CoA, 0.2 μmole NADPH and 42 μg protein. (•—•—•) Complete reaction mixture (NADPH added as indicated); (○—○—○) control (feruloyl-CoA omitted, but NADPH throughout the reaction).

incubated in the presence of reduced pyridine nucleotide and crude enzyme. However, under these conditions, feruloyl-CoA underwent rapid hydrolysis. Thus, the enzyme was further fractionated on a Sephadex G-200 column and it was possible to remove an apparent thiolesterase activity. A protein fraction was found which catalyzed the reduction of feruloyl-CoA at the expense of NADPH (fig. 2). As indicated, there was only minimal thiolesterase activity present and upon addition of NADPH a rapid decrease in optical density due to the specific reduction of feruloyl-CoA was observed. There was only negligible NADPH oxidase activity observed in this fraction. The reaction had an absolute specificity for NADPH and substitution of NADPH by NADH resulted in no reduction. The product of the reaction was identified by two ways as coniferyl aldehyde. First, tritium labelled feruloyl-CoA was reduced under the conditions given in fig. 2 in the presence of NADPH. The products of the reaction were extracted [1] and identified by chromatography as coniferyl aldehyde and alcohol. To confirm the occurrence of intermediate coniferyl aldehyde, a reaction mixture containing the reagents necessary for the overall reaction was incubated with crude enzyme in the presence of phenylhydrazine. A radioactive derivative (cf. Materials and methods) was found which upon chromatography in both solvent systems (solvent I: R_f 0.27; solvent II: R_f 0.59) was coincident with authentic material. It is therefore concluded that feruloyl-CoA reductase catalyzes the reduction of feruloyl-CoA to the aldehyde level with NADPH as reductant. Since cinnamoyl-CoA reductase functions at a branch point in phenylpropane metabolism [6], this enzyme may have important control functions. Studies regarding these regulatory properties are in progress.

3.3. *Coniferyl alcohol oxidoreductase*

As shown above, a combination of ferulate:CoA ligase and feruloyl-CoA reductase is involved in the formation of coniferyl aldehyde. This compound can undergo polymerisation during lignin formation [7] or may be reduced to coniferyl alcohol prior to polymerisation. This latter step would necessarily involve a cinnamyl aldehyde reductase.

Such an enzyme activity was found in the crude extracts. An optical assay based upon the strong absorption at 400 nm of coniferyl aldehyde in basic solution was used. By this method, coniferyl alcohol oxidoreductase activity could easily be demonstrated in the enzyme extract (table 2). The production of coniferyl alcohol from the aldehyde was confirmed as previously reported [1]. The enzyme was specific for NADPH and the reaction was readily reversible. Its substrate specificity is yet unknown, however, it is most likely that this enzyme is specific for converting cinnamyl aldehydes into the immediate lignin precursors. It belongs to the large group of aromatic alcohol oxidoreductases (cf. [8, 9]).

4. Discussion

The results presented in this paper show that feruloyl-CoA is converted into coniferyl alcohol in a two step reduction involving coniferyl aldehyde as an intermediate. In the course of this study feruloyl-CoA has been established for the first time as a natural intermediate in the biosynthesis of the most abundant lignin precursor in higher plants. Previously, only indirect evidence has been obtained for the formation of cinnamoyl-CoA esters in higher plants [10, 11].

Based on preliminary experiments, the overall reaction catalyzed by these enzymes can be summarized as follows:

Table 2
Cofactor requirement for coniferyl alcohol oxidoreductase.

System A	Coniferyl aldehyde consumed (nmole·min^{-1})	System B	Coniferyl alcohol oxidized (nmole·min^{-1})
Complete	10.6	Complete	5.3
minus NADPH	0.6	minus $NADP^+$	0
minus NADPH, plus NADH	1.3	minus $NADP^+$, plus NAD^+	0
minus enzyme	1.2	minus enzyme	0

System A: The complete reaction mixture contained in a final volume of 0.5 ml 50 μmoles Tris-HCl, pH 8.0, 23 nmoles coniferyl aldehyde, 60 nmoles NADPH and 0.13 mg protein. *System B:* The complete reaction mixture contained in a final volume of 0.5 ml 50 μmoles borate buffer, pH 9.0, 60 nmoles coniferyl alcohol, 80 nmoles $NADP^+$ and 0.13 mg protein. The assays were followed spectrophotometrically at 400 nm (temperature 30°). Rates of reaction were calculated using calibration curves for coniferyl aldehyde at the appropriate pH.

$$R{-}CH{=}CH{-}COOH + ATP + CoA\text{-}SH \rightarrow R{-}CH{=}CH{-}CO \sim S\cdot CoA + AMP + PP_i$$

$$R{-}CH{=}CH{-}CO \sim S\cdot CoA + NADPH + H^+ \rightarrow R{-}CH{=}CH{-}CHO + CoA\text{-}SH + NADP^+$$

$$R{-}CH{=}CH{-}CHO + NADPH + H^+ \rightleftharpoons R{-}CH{=}CH{-}CH_2OH + NADP^+$$

(R = 4-hydroxy-3-methoxyphenyl)

This reaction sequence for the reduction of the carboxyl group of phenylpropanoic acids to form primary alcohols of the cinnamyl alcohol type is quite different from the one found in fungi, since the latter does not involve CoA-thiolesters [12].

The possibility that sulfhydryl esters, perhaps of the CoA type, are involved in the reduction of cinnamic acids during lignin biosynthesis was, to our knowledge, first suggested by Brown et al. [13] in 1959.

With the findings presented here one of the "most important gaps remaining in our knowledge of lignification chemistry" [14] is at least partially filled.

Acknowledgements

Our thanks are due to Miss W. Bünger for excellent technical assistance and to the "Bundesminister für Bildung und Wissenschaft, Bonn" for financial support.

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