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SYNTHESIS OF *p*-COUMAROYL COENZYME A WITH A PARTIALLY PURIFIED *p*-COUMARATE:CoA LIGASE FROM CELL SUSPENSION CULTURES OF SOYBEAN *(GLYCINE MAX)*

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SUMMARY

1. A *p*-coumarate:CoA ligase has been isolated from cell suspension cultures of soybean. Partial purification of the enzyme was achieved by $MnCl_2$ and $(NH_4)_2SO_4$ precipitation and by gel chromatography on Sephadex G-100.

2. Under the conditions of the hydroxamic acid assay, the enzyme has a pH optimum at about pH 8.5 and a temperature optimum at about 30 °C. It requires Mg^{2+} and ATP at a molar ratio of 1:1. The molecular weight of the enzyme was estimated from the elution volume after chromatography on a Sephadex G-100 column to be about 55 000.

3. Of the various cinnamic acids tested, *p*-coumarate was the most effective substrate.

4. The partially purified *p*-coumarate:CoA ligase was utilized for the enzymatic synthesis of *p*-coumaroyl CoA. Gel chromatography on Sephadex G-10 proved to be a simple and rapid procedure for the isolation and purification of the product.

INTRODUCTION

The formation of CoA thiol esters has been postulated to be involved in the conversion of cinnamic acids to flavonoids[1], lignin[2], benzoic acids[3], and other products in higher plants. Recently, we reported the enzymatic formation of naringenin (5,7,4′-trihydroxyflavanone) from *p*-coumaroyl CoA and malonyl CoA in extracts from cell suspension cultures of parsley *(Petroselinum hortense)*[4]. For these investigations, it was necessary to synthesize radioactive and non-labelled *p*-coumaroyl CoA.

Gross and Zenk[2] described the synthesis of the CoA thiol esters of a number of substituted cinnamic acids with an enzyme preparation from beef liver mitochondria. This enzyme (acid:CoA ligase, EC 6.2.1.2) is not specific for cinnamic acids, but acts

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on a large variety of acids from C_4 to C_{11} including unsaturated and aromatic acids[5,6].

Walton and Butt[7] reported the formation of cinnamoyl CoA in extracts from leaves of spinach beet *(Beta vulgaris)*. Enzymes specifically involved in the metabolism of substituted cinnamic acids in higher plants have recently been demonstrated in cell suspension cultures of parsley[8] and of soybean[9]. In particular, soybean cell cultures exhibited high specific activities of *p*-coumarate:CoA ligase at certain stages during the growth cycle[9]. The present communication reports the partial purification and some properties of this enzyme as well as its utilization for the synthesis of *p*-coumaroyl CoA. A simple and rapid method for the isolation and purification of the reaction product is described.

MATERIALS AND METHODS

Materials

CoA (reduced coenzyme A) and ATP were purchased from Boehringer, Mannheim. *p*-[2-^{14}C]Coumaric acid was a gift from Dr J. Ebel, Freiburg.

Soybean cells

Cell suspension cultures of *Glycine max* were grown in 2-l Erlenmeyer flasks as described elsewhere[10]. The precise growth phase in which the cells reached maximum *p*-coumarate:CoA ligase activity was determined by measuring the conductivity of the culture medium[10]. Cells were harvested at this stage by vacuum filtration through a porous glass filter, and were either used immediately for enzyme extraction or frozen with liquid nitrogen and stored at −20 °C. No significant loss of *p*-coumarate:CoA ligase activity was observed during storage of the cells for several months at this temperature.

Standard enzyme assay

A mixture containing 5 μmoles of *p*-coumarate, 1 μmole of CoA, 3 μmoles of ATP, 3 μmoles of $MgSO_4$, 200 μmoles of hydroxylamine, and 200 μmoles of KCl in a total volume of 125 μl was adjusted to pH 8.0 with 1 M KOH and incubated with 0.1–0.4 mg of protein in 100 μl of 20 mM potassium phosphate (pH 7.5, containing 1 mM dithioerythritol) at 30 °C for 45 min. The reaction was stopped by adding 300 μl of an acidic solution of $FeCl_3$ (ref. 11) to the mixture. Denatured protein was removed by centrifugation, and the absorbance at 546 nm was measured against a blank containing no *p*-coumarate. Amounts of hydroxamic acid formed were calculated from the extinction coefficient for cinnamoyl hydroxamate ($1.54 \cdot 10^6$ cm^2/mole) determined by Gross and Zenk[2]. 1 unit of activity is defined as the amount of enzyme which catalyzes the formation of 1 nmole of hydroxamic acid in 1 h at 30 °C in the standard assay. The reaction product of the hydroxamic acid assay was identified as described in a previous communication[8].

Enzyme purification

All steps were carried out at 4 °C. 220 g of cells were homogenized for 5 min in a chilled mortar with 110 g of quartz sand and 220 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 1 mM mercaptoethanol. After centrifugation for 15 min at 15 000 × *g*, the supernatant was stirred for 20 min with 16 g of Dowex

1-X 2 (phosphate form, equilibrated with 0.2 M potassium phosphate, pH 7.5) and filtered through glass wool. A solution of 1 M $MnCl_2$ was slowly added to the filtrate to give a final concentration of 50 mM $MnCl_2$. After stirring for 20 min, the precipitate was removed by centrifugation. The supernatant was adjusted to 50% saturation by the addition of a concentrated solution of $(NH_4)_2SO_4$. The precipitate was again removed by centrifugation. The remaining protein was precipitated from the supernatant with solid $(NH_4)_2SO_4$ (80% saturation), centrifuged off, and resuspended in 10 ml of 20 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithioerythritol. This solution was stored frozen at −20 °C (Solution A). *p*-Coumarate:CoA ligase activity was stable under these conditions for several months.

7 ml of solution A (6.3 mg protein/ml) were passed through a Sephadex G-100 column (3 cm × 40 cm) with 50 mM potassium phosphate buffer (pH 7.5). Fractions of 5.2 ml each were collected and assayed for *p*-coumarate:CoA ligase activity. The combined Fractions 18–22 (Fig. 1) were used for determination of optimum conditions for the enzyme assay (Solution B).

Determination of the molecular weight

The molecular weight of the *p*-coumarate:CoA ligase was determined on the Sephadex G-100 column used by Sutter *et al.*[12].

Synthesis of p-coumaroyl CoA

Enzyme solution A (4.5 ml) was incubated with 40 μmoles of potassium *p*-coumarate, 20 μmoles of CoA, 120 μmoles of ATP, 120 μmoles of $MgSO_4$, and 300 μl of 1 M KOH at 30 °C for 90 min. For the synthesis of ^{14}C-labelled *p*-coumaroyl CoA, the incubation mixture contained 23 μmoles of *p*-[2-^{14}C]coumarate (1.85 Ci/mole). The reaction was stopped by adding 20 μl of formic acid (radioactive incubation) or adjusting the mixture to pH 3 by the addition of 50 mM formic acid and removing the denatured protein by centrifugation. The precipitate was twice extracted with 5 ml each of 50 mM formic acid and centrifuged. Free *p*-coumaric acid was removed from the combined supernatant fractions by extraction with diethyl ether (5 × 15 ml). The aqueous solution was concentrated *in vacuo* at 20 °C to 5 ml and passed through a column of Sephadex G-10 (3.6 cm × 39 cm) with 50 mM formic acid at 4 °C. Fractions of 6.7 ml each were collected, and the absorbance at 254 nm was automatically recorded. The ultraviolet spectra of the individual fractions were measured. Fractions 59–95 (Fig. 4) were combined and concentrated *in vacuo* to approximately 5 ml. This solution was stored at −20 °C. The overall yield of the purified thiol ester, based on the initial amount of CoA, was about 10%.

Determination of radioactivity

This was carried out with a Beckman LS 100 scintillation spectrometer using a solution of PPO (5 g/l) and naphthalin (100 g/l) in dioxane. Counting efficiency (90%) was determined by internal standardization.

Protein assay

Protein was determined by the biuret or the Lowry method[13].

TABLE I

PARTIAL PURIFICATION OF *p*-COUMARATE:CoA LIGASE

Purification step	*Protein (mg)*	*Specific activity (munits/mg protein)*	*Purification (-fold)*
Crude extract	900	—*	—
Dowex supernatant	600	—*	—
$MnCl_2$ fractionation	135	—*	3**
$(NH_4)_2SO_4$ fractionation	63	14	5
Sephadex G-100 column	30	40	12

* Enzyme activity could not be determined by the hydroxamic acid assay in extracts containing 0.2 M phosphate.

** Based on a result obtained when 0.2 M Tris–HCl buffer, pH 7.5, was used instead of potassium phosphate[14].

RESULTS

Partial purification and some properties of p-coumarate:CoA ligase

Table I summarizes the results of an enzyme purification procedure including treatment of a crude extract with Dowex 1, fractionation with $MnCl_2$ and $(NH_4)_2SO_4$, and Sephadex G-100 column chromatography. After the $(NH_4)_2SO_4$ step, the enzyme could be stored at −20 °C for several weeks without significant loss of activity. However, it was very unstable at 4 °C in potassium phosphate, Tris–HCl or glycine–KOH buffers containing 1 mM dithioerythritol and lost all of its activity within a few days. In the absence of thiol reagents the enzyme was even less stable. For this reason, further attempts to purify the enzyme beyond the gel chromatography step have thus far been unsuccessful.

Data given in Table I for the extent of purification certainly represent minimum values because of the instability of the enzyme during the purification procedure. The extent of enzyme purification by $MnCl_2$ fractionation was calculated from an experiment with Tris–HCl buffer. When the first three steps of purification were carried out in 0.2 M Tris–HCl buffer at pH 7.5 (in which the enzyme is less stable than

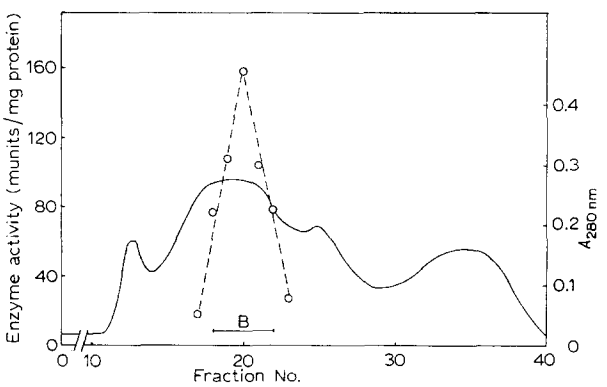

Fig. 1. Purification of *p*-coumarate:CoA ligase (○---○) by chromatography on a Sephadex G-100 column. ———, protein.

TABLE II

ENZYME ACTIVITY MEASURED IN THE STANDARD ASSAY AT DIFFERENT MOLAR RATIOS OF ATP AND Mg^{2+}

Expt No.	*ATP (μmoles)*	*Mg^{2+} (μmoles)*	*Relative enzyme activity*
1	3	1	65
	3	3	100
	3	6	75
2	1	3	60
	3	3	100
	6	3	78

in the phosphate buffer normally used) a three-fold increase in the specific activity of *p*-coumarate:CoA ligase was achieved by the $MnCl_2$ fractionation. The $(NH_4)_2SO_4$ fractionation (under the standard conditions described in the experimental section) resulted in a recovery of approx. 75% of the enzyme activity in the second fraction (50–75% saturation)[14]. Gel chromatography on Sephadex G-100 was used as a final purification step resulting in an approximately 12-fold purification of the enzyme. The combined Fractions 18–22 (Solution B as indicated in Fig. 1) were used to determine optimum assay conditions and some substrate specificities.

Highest specific activities of *p*-coumarate:CoA ligase in Solution B were obtained when the assay mixture contained the amounts of reagents described for the standard incubation. At molar ratios of ATP and Mg^{2+} higher or lower than 1:1, enzyme activity was reduced (Table II). No enzyme activity was found in the absence of either Mg^{2+}, ATP, or CoA.

The pH optimum was at about pH 8.5 (Fig. 2), the temperature optimum at 30 °C (Fig. 3).

The formation of hydroxamic acids was linear with time for at least 45 min at protein concentrations up to at least 0.43 mg per assay.

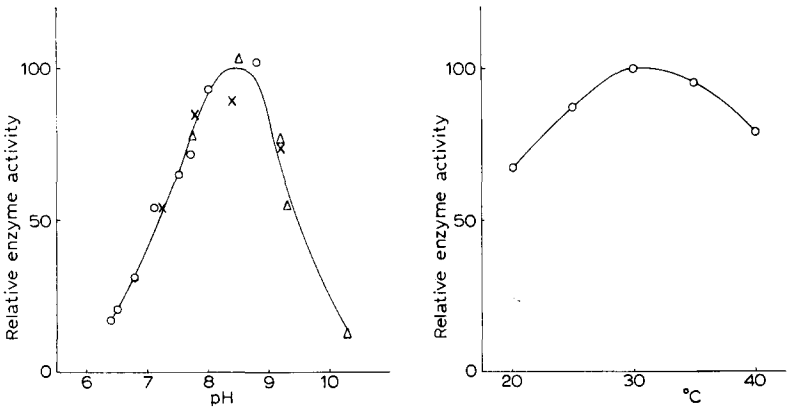

Fig. 2. Dependence of enzyme activity on pH. ○, potassium phosphate buffer; ×, Tris–HCl buffer; △, glycine–KOH buffer.

Fig. 3. Dependence of enzyme activity on temperature. Assay conditions were as defined under Materials and Methods.

TABLE III

COMPARISON OF VARIOUS CINNAMIC ACIDS AS SUBSTRATES OF *p*-COUMAROYL:CoA LIGASE IN THE STANDARD ASSAY CONTAINING 6 μMOLES OF THE RESPECTIVE CINNAMIC ACID

nmoles of products formed were determined by using the extinction coefficient for cinnamoyl hydroxamic acid[2].

Acid	*Hydroxamic acid formed (nmoles)*	*%*
p-Coumaric	134	100
Ferulic	66	49
Caffeic	20	14
p-Methoxycinnamic	16	12
Cinnamic	13	10

The substrate specificity of the enzyme towards five cinnamic acids is shown in Table III. Although their chemical structures are closely related to *p*-coumaric acid, ferulic, caffeic, *p*-methoxycinnamic, and cinnamic acids were much less effectively converted than *p*-coumarate.

An estimation of the molecular weight of the *p*-coumarate:CoA ligase was obtained by determination of the elution volume on a calibrated Sephadex G-100 column. A value of about 55 000 was calculated for this enzyme.

Synthesis and purification of p-coumaroyl CoA

An enzyme preparation purified to the $(NH_4)_2SO_4$ fractionation step (Solution A) was used for the synthesis of *p*-coumaroyl CoA. The isolation of the product by gel filtration on a Sephadex G-10 column is illustrated in Fig. 4. The CoA thiol ester was completely separated from other compounds absorbing at 254 nm (CoA, ATP). The ultraviolet spectrum of the combined Fractions 59–95 (see Fig. 4) was identical with that previously described for *p*-coumaroyl CoA with characteristic absorption peaks at 333 nm and 259 nm (ref. 2).

When the isolated product was treated with 0.4 M KOH for 4 min and subsequently acidified to pH 3 with formic acid, the absorption peak at 333 nm had disap-

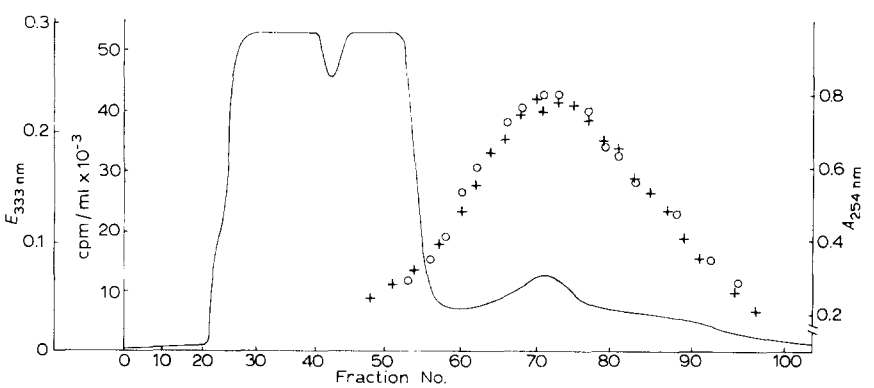

Fig. 4. Purification of *p*-[2-^{14}C]coumaroyl CoA by chromatography on a Sephadex G-10 column. ———, absorbance at 254 nm; ○, extinction at 333 nm; +, radioactivity.

peared, and a new peak characteristic of the ultraviolet spectrum of *p*-coumaric acid was observed at 310 nm. Further evidence for the identity of the isolated compound was obtained when ^{14}C-labelled *p*-coumarate was used as a substrate of the enzyme. Both radioactivity and extinction measured at 333 nm coincided with the peak recorded at 254 nm in the eluate from the gel column (Fig. 4).

From the radioactivity and from the extinction coefficient of *p*-coumaroyl CoA at 333 nm (ref. 2) an overall yield of approx. 10%, based on the amount of CoA converted, was calculated. Starting with 100 g of cells, 2 mg of *p*-coumaroyl CoA were synthesized and purified within 24 h.

DISCUSSION

This report describes the partial purification of an enzyme catalyzing the formation of CoA thiol esters of cinnamic acids in higher plants for the first time. Although the *p*-coumarate:CoA ligase from soybean cell cultures is very unstable under the conditions of the purification procedure reported here, the enzyme can be stored frozen for several weeks without loss of activity.

In contrast to an enzyme from spinach beet reported to be "virtually specific" for unsubstituted cinnamic acid[7], the enzyme from soybean cell cultures exhibits a high degree of specificity for *p*-coumaric acid. Among the substrates tested, cinnamic acid was least effectively converted (Table III). Similar results have been described for a crude enzyme preparation from parsley cell cultures[8], while an enzyme from beef liver mitochondria shows little specificity for the substitution pattern of a number of cinnamic acids and is not specific for the metabolism of aromatic compounds[2]. Results presented in Table III and previous studies on concomitant changes in the activities of phenylalanine ammonia-lyase (EC 4.3.1.5) and *p*-coumarate:CoA ligase during the growth of soybean cell suspension cultures[9] suggest that the enzyme described in this paper is specifically involved in the metabolism of phenylpropanoid compounds.

The apparent molecular weight of the *p*-coumarate:CoA ligase is the same (within 10%) as those of several acetate:CoA ligases from potato tuber[15] and of three soluble enzymes involved in phenylpropanoid metabolism in parsley cell cultures, a glucosyltransferase[12], an apiosyltransferase[12], and a methyltransferase[16].

A very rapid and simple procedure was developed for the enzymatic synthesis and the purification of *p*-coumaroyl CoA. In contrast to the method described by Gross and Zenk[2] which includes a time-consuming step of paper chromatography and subsequent elution of the CoA thiol ester, the procedure reported here allows the synthesis of several milligrams of *p*-coumaroyl CoA in 1 day. An additional advantage is the stability of the *p*-coumarate:CoA ligase when either the intact cells or the partially purified enzyme are kept frozen. On the other hand, there are only a limited number of substituted cinnamic acids of which CoA thiol esters can be synthesized in good yield with the *p*-coumaroyl:CoA ligase from soybean cell suspension cultures.

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