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FACILE ENZYMIC SYNTHESIS OF CAFFEOYL CoA

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Key Word Index—*Populus tremuloides*; aspen; caffeoyl CoA; *p*-hydroxycinnamic acid:CoA ligase; lignin; *O*-methyltransferase.

Abstract—*p*-Hydroxycinnamic acid:CoA ligase (EC 6.2.1.12) was extracted from aspen xylem and its activity with several cinnamic acids was determined. The highest activity was found with *p*-hydroxycinnamic acid and the extract had reasonable activity with ferulic, caffeic and 5-hydroxyferulic acids but none with sinapic acid. Using this isolated enzyme preparation, caffeoyl CoA was synthesized and subsequently purified using reversed phase C-18 solid phase extraction. This enzymic synthesis method, which yielded 40% product, is rapid and simple and provides a cost-effective alternative to the use of commercial acyl CoA ligase. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

The interest in the lignin biosynthetic pathway and its biotechnological manipulation is rapidly growing because lignin is one of the main obstacles to the optimal use of biomass [1–3]. Since lignins with a higher degree of methoxylation of the phenolic parental units have higher rates of chemical breakdown [4], considerable attention has been focused on the *O*-methylation reactions in monolignol biosynthesis and the *O*-methyltransferase (OMT) enzymes catalyzing these reactions. Originally, caffeic/5-hydroxyferulic acid OMT was thought to be the only enzyme catalyzing methylation steps in monolignol biosynthesis [5, 6]. However, recently caffeoyl CoA OMT, which was discovered playing a role in plant defense response [7], has been found to catalyse O-methylation steps in general monolignol biosynthesis [8, 9]. The fact that caffeoyl CoA is not commercially available hinders the study of the physiological role of this newly recognized OMT form.

The synthesis of the CoA thioester substituted cinnamic acids by chemical methods has been reported [10]. Considering that the yields were low and that process was time consuming and complex, we investigated an enzymic method to synthesize caffeoyl CoA. The method we have developed is a modification of enzymic methods previously developed for synthesis of fatty acyl-CoA derivatives using acyl CoA ligases from animal mitochondria and a bacterium [11, 12]. Since the commercially available bacterial acid CoA ligase is not specific for cinnamate derivatives and provides low yields in the coupling of these substrates to CoA, we isolated *p*-hydroxycinnamic acid:CoA ligase from aspen xylem for use in synthesis of caffeoyl CoA. Here we report the characterization of the crude preparation of *p*-hydroxycinnamic acid:CoA ligase from aspen xylem and its application for the rapid synthesis of caffeoyl CoA, which is demonstrated to be an effective substrate for recombinant aspen xylem caffeoyl CoA OMT.

RESULTS AND DISCUSSION

Characterization of p*-hydroxycinnamic acid:CoA ligase from aspen xylem.*

p-Hydroxycinnamic acid:CoA ligase has been isolated and characterized from several plant species and xylem has been shown to be a good source of the enzyme compared to other parts of the plant [13–15]. Therefore, in this study, developing secondary xylem tissue from aspen was used as the source for this enzyme. The isolated crude CoA ligase was tested using several cinnamic acids as substrates (Table 1). *p*-Hydroxycinnamic acid gave the highest activity, while activity levels about two-thirds as high were found with ferulic and caffeic acids. The activity with 5-hydroxyferulic acid was much lower and there was no activity with sinapic acid. This substrate specificity profile for aspen CoA ligase is similar to that for *p*-hydroxycinnamate:CoA ligases isolated from pine [13], willow [17], wheat [18] and many other higher plants

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Table 1. Substrate profile of *p*-hydroxycinnamic acid:CoA ligase from aspen xylem. The enzyme was prepared as described in the Experimental section

Substrate (0.2 mM)	Activity (pkat/mg)	Relative activity (%)
p-Hydroxycinnamic acid	260	100
Ferulic acid	175	67
Caffeic acid	160	61
5-Hydroxyferulic acid	107	41
Sinapic acid	0	0

[14, 16]. But it differs from the CoA ligase from poplar studied by Grand *et al.* [15]. They found there were several isozymes of CoA ligase in *Populus* xylem, however, 75% of the activity in xylem was present as the so-called 'Form I', which was most active with *p*-hydroxycinnamic acid like the enzyme preparation isolated here. Strikingly, the Form I CoA ligase had no activity with caffeic acid and a high activity with sinapic acid [15], both of which contrast strongly with our results.

Caffeoyl CoA synthesis and purification.

In order to optimize the synthesis of caffeoyl CoA using the *p*-hydroxycinnamic acid:CoA ligase extracted from aspen xylem, the optimal pH of this enzyme with caffeic acid as substrate was examined and found to be pH 7.5. As it has been reported that high concentration of cinnamic acids inhibit the enzyme [17], the optimal concentration of caffeic acid was studied (Fig. 1). The highest activity was found at caffeic acid concentration of 0.2 mM, when CoA concentration was varied from 0.05 to 0.2 mM. Since 0.1 mM CoA gave nearly the same initial reaction rate as 0.2 mM CoA and CoA is a much more costly substrate than caffeic acid, the conditions for the bulk synthesis of caffeoyl CoA were set to 0.1 mM CoA and 0.2 mM caffeic acid. This concentration of CoA is much lower than the 5 mM level prescribed for the synthesis of acyl-CoA derivatives using bacterial acid CoA ligase [12].

To synthesize bulk amounts of caffeoyl CoA, much larger amounts of crude aspen *p*-hydroxycinnamic acid:CoA ligase were used and the reaction run for 5 hr at room temperature. Other substrates and additives used in the standard CoA ligase assay were held constant. The time course of the reaction was followed by monitoring the increase in absorbance at 346 nm to evaluate the synthesis of caffeoyl CoA (Fig. 2). These results showed that the reaction had come to near completion by about 3 hr. The addition of ATP, CoA, caffeic acid or enzyme at this time did not lead to a new increase in 346 nm absorbance, which may indicate the accumulation of products inhibited further reaction and accumulation of caffeoyl CoA. Furthermore, the effect of increasing the CoA concentration by 10-fold to 1 mM was evaluated and we found that this had little impact on the total amount of caffeoyl CoA synthesized.

At the end of the 5 hr reaction time, the mixture was directly loaded to the reversed phase solid extraction column which has been equilibrated with 0.2 M Mops, pH 7.5. Under these conditions, polar compounds such as unreacted caffeic acid and CoA do not bind to the column while caffeoyl CoA binds because it is relatively less polar. After extensive washing the column with equilibrium buffer (about 25 ml) until the effluent had no absorbance at 257 nm, deionized water was used to elute the caffeoyl CoA. After about 1 or 2 column volumes had passed through the column, caffeoyl CoA started to elute as indicated by its light-yellow colour. The complete elution of caffeoyl CoA required about 30 column volumes of deionized water or about 30

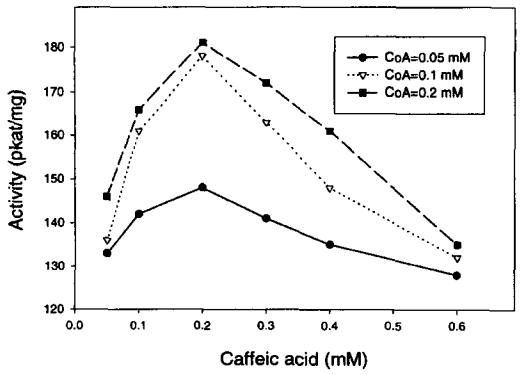

Fig. 1. Optimal caffeic acid concentration for the *p*-hydroxycinnamic acid:CoA ligase catalysed reaction with 0.05, 0.1 and 0.2 mM CoA.

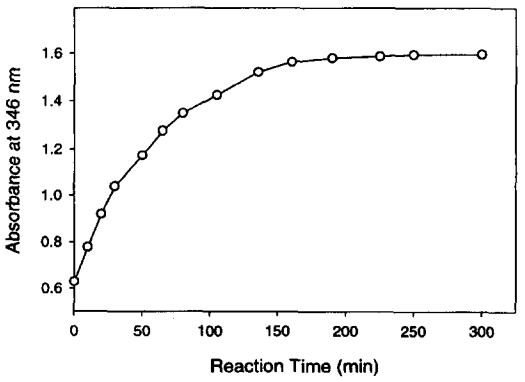

Fig. 2. Bulk enzymic synthesis of caffeoyl CoA catalysed by aspen xylem *p*-hydroxycinnamic acid:CoA ligase. The increase of absorbance at 346 nm was monitored during the reaction.

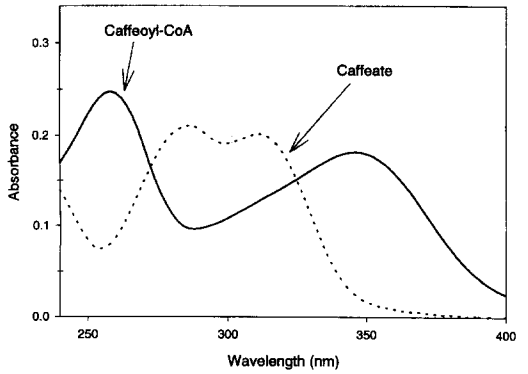

Fig. 3. Absorbance spectra of caffeoyl CoA and caffeic acid. Authentic caffeic acid (0.014 mM) or enzymically synthesized and purified caffeoyl CoA (0.01 mM) in 30 mM Mops, pH 7.0, were scanned in an HP8452A Diode Array UV–Vis Spectrophotometer.

ml, which was pooled and concentrated in a vacuum centrifuge. TLC analysis showed the purified product was pure and free from unreacted caffeic acid, CoA and ATP and had the same R_f as chemically synthesized caffeoyl CoA (results not shown). The spectrum of the enzymically synthesized and purified caffeoyl CoA (Fig. 3) is essentially identical to that previously reported [10]. The two peaks at 257 and 346 nm are characteristic of caffeoyl CoA and the spectrum of the thioester derivative is quite different from caffeate (Fig. 3). Based on the absorbance at 346 nm, the total yield of caffeoyl CoA was about 40% of the amount of CoA started with in the bulk reaction.

We have cloned both caffeic acid OMT and caffeoyl CoA OMT cDNAs from aspen xylem and expressed them in *Escherichia coli* [5, 9, 19]. Therefore both enzymes were used to test the enzymically synthesized caffeoyl CoA (Table 2). Enzymically synthesized caffeoyl CoA was found to be as effective as chemically synthesized caffeoyl CoA as a substrate for these OMTs. While the recombinant aspen xylem caffeoyl CoA OMT uses caffeic acid as a substrate, the free acid is only about 4% as active as the thioester of caffeic acid, which indicates the importance of the large side chain ester in substrate binding to the enzyme. In contrast, caffeic acid OMT, which also uses both these compounds as substrate [19], has only 25% as much activity with caffeoyl CoA as substrate as compared to caffeic acid (Table 2).

Summary.

A simple method for preparation of a crude form of aspen xylem *p*-hydroxycinnamic acid: CoA ligase has been developed. The enzyme preparation can be stored at −20° for a period of two to three months. Aspen xylem CoA ligase is most active with *p*-hydroxycinnamic acid, but is also highly active with caffeic acid as substrate. The crude aspen CoA ligase can be effectively and economically used to synthesize caffeoyl CoA. A simple and rapid purification method was used for product purification, which greatly enhances the utility of the enzymic method for synthesis of caffeoyl CoA. The caffeoyl CoA was shown to have the same spectrum as was previously published for chemically synthesized and purified caffeoyl CoA and to be an effective substrate for recombinant aspen xylem caffeoyl CoA OMT. Because most *p*-hydroxycinnamate:CoA ligases isolated so far from various higher plants showed good activity toward caffeic acid [13, 14, 17, 18], it is expected *p*-hydroxycinnamate:CoA ligases from plants other than aspen may also be used for the synthesis of caffeoyl-CoA. The availability of a simple method for the preparation of caffeoyl CoA should make it much easier to characterize lignin-specific forms of caffeoyl CoA OMT and investigate the important of this enzyme form in the biosynthesis of monolignols in xylem of woody species.

EXPERIMENTAL

p-Hydroxycinnamic acid:CoA ligase extraction. Aspen (*Populus tremuloides*) developing secondary xylem (7 g), collected in June 1995 and stored in liquid nitrogen, was ground with sand in a mortar with liquid nitrogen. The powdered tissue was transferred to a blender and 50 ml of extraction buffer (0.1 M Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 2 g polyvinylpolypyrrolidone and 25% glycerol) was added. The mixture was blended for 2 min at high speed and filtered through 4 layers of cheesecloth. The filtrate was centrifuged at 20000 *g* for 15 min and the supernatant was used for enzyme assays or stored at −20° for later use.

p-Hydroxycinnamic acid:CoA ligase activity assay. The assay mixture (1 ml) contained 0.2 M Mops, pH

Table 2. Activities of crude recombinant aspen xylem caffeoyl CoA OMT and partially purified recombinant aspen xylem caffeic acid OMT using different substrates. The enzyme assay was carried out as described in the Experimental section

Substrate (0.1 mM)	Caffeoyl CoA OMT activity (pkat/mg)	Caffeic acid OMT activity (pkat/mg)
Enzymically synthesized caffeoyl CoA	343	1270
Chemically synthesized caffeoyl CoA	342	1293
Caffeic acid	15	5000
Control (H_2O)	0	0

7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM cinnamic acid or related substrate, 2.5 mM ATP, 100 μl enzyme solution and 0.1 mM CoA (added last to start the reaction). After addition of CoA, the cuvette was placed in the spectrophotometer (Shimadzu UV-1201) and the increase of absorbance at λ_{max} of cinnamoyl thioester of interest was monitored. The following substrates and corresponding CoA ester product λ_{max} and extinction coefficients were used: *p*-hydroxycinnamate, 333 nm, 21 mM^{-1}; caffeate, 346 nm, 18 mM^{-1}; ferulate, 346 nm, 19 mM^{-1}, 5-hydroxyferulate, 350 nm, 20 mM^{-1}; and sinapate, 352 nm, 20 mM^{-1} [10, 13]. Control assays contained no CoA.

Caffeoyl CoA synthesis and purification. The caffeoyl CoA synthesis reaction (10 ml) was performed in a test tube containing the following components at final concentrations: 0.2 mM caffeic acid, 0.1 mM CoA, 2.5 mM ATP, 10 mM $MgCl_2$, 1 mM DTT, 0.2 M Mops, pH 7.5, and 2 ml crude *p*-hydroxycinnamic acid:CoA ligase extract containing 2 mg of protein. The reaction was allowed to proceed at room temperature for 5 hr and then the reaction mixture was applied directly to a disposable SPE LC-18 column (Supelco, bed volume 1 ml). Prior to applying the reaction mixture, the column had been washed with 3 column vol of methanol and equilibrated with 3 vol of 0.2 M Mops, pH 7.5. After sample application, the column was washed with 0.2 M Mops, pH 7.5, until the effluent had no absorbance at 257 nm. The caffeoyl CoA was then eluted with 30 ml deionized H_2O and concentrated in a vacuum centrifuge to a final volume of 0.8 ml.

Characterization of caffeoyl CoA. The identification and purity of caffeoyl CoA was determined by TLC (Silica Gel 60 F_{254}, EM Science) with caffeoyl CoA, synthesized by the chemical method [10], using *n*-butanol/water/acetic acid (50/30/20) as the solvent system. It was also characterized by spectral analysis (Hewlett Packard HP8452A Diode Array UV–Vis Spectrophotometer). Furthermore, enzyme assays were carried out using the recombinant aspen xylem caffeoyl CoA OMT and caffeic acid OMT expressed in *E. coli* [9]. Caffeoyl CoA OMT activity or caffeic acid OMT activity was assayed using the following mixture (50 μl): 50 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM substrates, 20 μl enzyme solution and 1 mM ^4C labelled *S*-adenosyl-L-methionine. After 10 min reaction at 30°, 5.5 μl 5 N NaOH was added to stop the reaction and the mixture was kept at 40° for 10 min to hydrolyse CoA thioester. Then 6.2 μl 6 N HCl was added, the mixture was extracted with 1 ml ethyl ether and the organic phase was counted for radioactivity, as previously described [20]. For caffeic acid OMT, the reaction mixture was acidified and extracted directly without incubation at 40° [20].

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