Enzymatic Synthesis and Purification of Caffeoyl-CoA, \( p \)-Coumaroyl-CoA, and Feruloyl-CoA

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An enzyme preparation from wheat seedlings containing \( p \)-coumaroyl:CoA ligase activity was used to synthesize caffeoyl-CoA, \( p \)-coumaroyl-CoA, and feruloyl-CoA. The same enzyme preparation also contains caffeic acid-3-O-methyl transferase and caffeoyl-CoA-3-O-methyl transferase activities. The maximum activity was found in enzyme preparation from 2-day-old seedlings, where 15–20% of the hydroxy cinnamic acid could be converted into the corresponding thioester. This yield is a result of an equilibrium between the ligase and a thioesterase also present in the crude enzyme preparation. The activity of caffeic acid 3-O-methyl transferase and caffeoyl-CoA 3-O-methyl transferase enables the production of \(^{14}C\)-labeled feruloyl-CoA when using \( S \)-adenosyl-L-[methyl-\(^{14}C\)]-methionine as methyl donor. The produced thioesters can be purified by reverse phase HPLC using a phosphoric acid-acetonitrile gradient.

Plant cells are surrounded by a strong wall, which serves to shape and support the plant and which presents a barrier against invading microorganisms. The cell wall is a complex and dynamic structure consisting mainly of polysaccharides and some protein. Cells that are no longer expanding may deposit a secondary wall that can be lignified. Lignin is a polymer that is very resistant to chemical and enzymatic breakdown. The precursors for lignin are alcohols derived from the hydroxy cinnamates \( p \)-coumaric, ferulic, and sinapic acid. Lignin is a product of oxidative polymerization of these alcohols known as monolignols. Ferulic and \( p \)-coumaric acid are not only precursors for lignin but are also found in the cell walls as acids ester-linked to polysaccharides. In xyloglucan, ferulic acid may be 4-O-ester-linked to xylopyranose (1). In arabinoxylan of grasses, ferulic acid and \( p \)-coumaric acid are 5’-linked to arabinoxylanoside (2). In pectin of some plants the acids are also linked to arabinofuranose but in this case the linkage is to the 2’ or 3’ (2, 3). In pectin, ferulic acid can also be ester-linked to galactose (4).

The biological significance of the ester-linked hydroxy cinnamates is poorly understood. Good evidence exists that ester-linked hydroxy cinnamates can be cross-linked to the lignin network (5, 6) and this is likely to protect the polysaccharide against degradation by herbivores and microorganisms (7–9). Hydroxy cinnamate esters in the wall may not only play a structural role, since esterified oligosaccharides have been shown to possess a signaling function in rice (10). Hydroxy cinnamates such as ferulic and caffeic acid have an antioxidant effect and have been implicated in the lowered risk of cancer correlated with a diet rich in whole-grain products (11, 12). The biosynthetic pathways for the formation of hydroxy cinnamates and monolignols have in general been elucidated although some variations between plants exist (13). Monolignols are formed by reduction of the corresponding thioesters, i.e., \( p \)-coumaroyl-, feruloyl-, and sinapoyl-CoA (13). In contrast, how the polysaccharide-hydroxy cinnamate esters are formed is unknown. The presumed activated precursors for incorporation of ferulic and \( p \)-coumaric acid into wall polysaccharides are hydroxy cinnamoyl-CoA thioesters (14, 15). Lignin biosynthesis or polymerization takes place in the wall. In contrast, the esterification of wall polysaccharides appears to occur intracellularly, presumably in the Golgi apparatus (14).

In order to investigate the biosynthesis of the esterified wall polysaccharides, it is necessary to have avail-
able the thioesters in both nonradioactive and radioactiv- 

cative form, but these compounds are not commercially available. Until now the hydroxy cinnamoyl thioesters have been produced chemically according to the procedure of Stöckigt and Zenk (16). We report here a single step enzymatic method for production of caffeoyl-CoA, p-coumaryl-CoA, and feruloyl-CoA, and we investigate the synthesis of $^{14}$C-feruloyl-CoA. The method is based on reports of caffeoyl 3-O-methyltransferase (17, 18), caffeoyl-CoA 3-O-methyltransferase (18), and p-coumaric acid:CoA ligase (19) activities. The reported optimal conditions for these enzymes are similar. We decided to investigate the possibility of obtaining the enzymes from a readily available source and of using them simultaneously in order to produce feruloyl-CoA in a single step with caffeic acid as starting material and S-adenosyl-$L-\left[{^{14}}C\right]$methyl][methyl-$^{14}C$]-methionine ($^{14}$C-SAM$^2$) as substrate for the $^{14}$C incorporation.

MATERIALS AND METHODS

Chemicals and Reagents

Chemicals and reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. S-Adenosyl-$L-\left[{^{14}}C\right]$methionine, $^{14}$C-SAM with a specific activity of 2.18 GBq/mmol was purchased from Amersham Life Science (Little Chalfont, UK), and acetonitrile was super purity solvent grade from Romil (Cambridge, England).

Plant Material

Seedlings of wheat (Triticum aestivum L. cv. Cad- 

denza) were grown in trays of vermiculite at 25°C for 2 to 9 days depending on the experiment. Unless other- 

wise indicated 2-day-old seedlings were used. The seedlings were grown in greenhouse with supplemen- 

tal light under a regime of 16 h light and 8 h dark.

Preparation of Enzymes

The procedure was modified from published methods for purification of caffeoyl-CoA ligase (19, 20) and O-methyltransferases (17, 19). The preparation was con- 

ducted at 4°C. Shoots and coleoptiles were harvested with a scalpel and ground with a mortar and pestle in an extraction buffer (2 ml per g of plant material) containing 0.05 M K$_2$HPO$_4$ (pH = 7.2), 0.4 M sucrose, 2.5 mM MgCl$_2$, and 0.6 mM DTT. The extract was filtered through a nylon mesh and centrifuged 10 min at 9,000g. This centrifugation step is important to minimize thioesterase activity (21). The resultant su-

pertanat was centrifuged 1 h at 48,000g to pellet the microsomes. The supernatant containing the cytosolic enzymes was subjected to (NH$_4$)$_2$SO$_4$ precipitation and the precipitate formed at 35–75% saturation was re-disolved in extraction buffer (1 ml per g of plant ma-

terial) and frozen in liquid N$_2$.

Synthesis of CoA Thioesters

The reactions were carried out in a vial (2 ml) contain- 

ning 0.2 M Mops (pH = 7.5), 10 mM MgCl$_2$, 1 mM DTT, 0.2 mM of the appropriate hydroxy cinnamic acid, 2.5 mM ATP, 0.2 mM CoA, 50 µl enzyme, and SAM in appropriate concentrations. The reaction was monitored spectrophotometrically at 346 nm in case of caffeoyl-CoA and feruloyl-CoA, and at 333 nm in case of p-coumaryl-CoA. The extinction coefficients used for quantification were $\varepsilon_{333} = 21$ mM$^{-1}$cm$^{-1}$ for p-cou- 

maroyl-CoA acid, $\varepsilon_{346} = 18$ mM$^{-1}$cm$^{-1}$ for caffeoyl-CoA acid, and $\varepsilon_{346} = 19$ mM$^{-1}$cm$^{-1}$ for feruloyl-CoA (16). When the reaction rate slowed down to 10% of the initial rate, 3 mg ATP was added and at the subse- 

quent decrease in reaction rate, 3 mg ATP, 8 mg phos- 

phocreatine, 150 units creatine phosphokinase, and 100 units myokinase were added. In some experi-

ments, 2 units of S-adenosyl homocysteine hydrolase were also added (see Results).

Anaerobic conditions were achieved in an anaerobic chamber with an atmosphere of N$_2$/CO$_2$/H$_2$ in the ratio 85:10:5, where the solutions were placed 1 day before use. The cuvette in which the reaction was monitored was sealed to prevent exposure to oxygen during the reaction. Samples were otherwise handled like under aerobic conditions.

Purification of CoA Thioesters

After incubation, the reaction vial was boiled for 5 

min and centrifuged 2 min at 15,000g in order to remove precipitated protein. The sample was subse- 

quently acidified to pH 3.0 with 1 M HCl and extracted four times with equal volumes of ethylacetate to re- 

move free cinnamic acids (22). The sample was then subjected to HPLC on a reverse-phase Nucleosil 10 C$_{18}$ column (250 × 4.6 mm Macherey-Nagel, Düren, Ger-

many). Prior to injection, the samples were spin fil-

tered (0.2 µm). For HPLC a flow of 1 ml/min and a 

gradient of solvent A (1% phosphoric acid) and solvent B (acetonitrile) were applied as follows: 0–5 min, 5% B isocratic; 5–25 min, 5–25% B linear; 25–40 min, 25– 

29% B linear; 40–41 min, 29–100% B linear; 41–50 

min, washing and reequilibration of the column. Peaks of interest were collected in 2-ml fractions.

To obtain the thioester in dry form a further HPLC purification was carried out using the same column,

$^2$Abbreviations used: SAM, S-adenosyl-$L$-methionine; $^{14}$C-SAM, S-adenosyl-$L-\left[{^{14}}C\right]$-methionine; TFA, trifluoroacetic acid.
but exchanging the solvents with solvent C (1 mM TFA and 10% (v/v) acetonitrile) and solvent D (1 mM TFA, 40% (v/v) acetonitrile and 40% (v/v) methanol) and applying a linear gradient of 10–100% D over 25 min to the column equilibrated with 10% D.

Characterization of Thioesters

The collected thioesters were neutralized with 1 M KOH and the characteristic spectra of cinnamoyl thioesters were recorded. Products were hydrolyzed in 1 M KOH at room temperature for 24 h and analyzed by HPLC applying the above described method with a solvent system of phosphoric acid and acetonitrile.

Table 1 shows recovered caffeoyl-CoA and caffeic acid. In the aerobic experiment much less of the total caffeic acid could be accounted for, but nevertheless the amounts of recovered thioester in the two experiments were the same. Since the yield of caffeoyl-CoA was the same in the two experiments it was decided to perform subsequent experiments under aerobic conditions, since this facilitates the experimental work considerably.

The large ΔA in the aerobic experiment and small recovery of total cinnamic acids indicate oxidative formation of dicaffeic acids. Consequently, the increase in absorbance was not solely due to the ligase reaction, but a good correlation exist between increase in absorbance and formation of hydroxy cinnamoyl-CoA as demonstrated in Fig. 1. In the experiment illustrated in Fig. 1, SAM was present in excess amounts and therefore feruloyl-CoA was the sole product, whereas caffeoyl-CoA was not detected.

Optimal Conversion by Adding Cofactors and Auxiliary Enzymes

To optimize the conversion of hydroxy cinnamic acids into thioesters the effect of adding ATP, phosphocreatine, creatine phosphokinase, myokinase, and S-adenosyl-L-homocysteine hydrolase was examined. Optimal conversion was achieved by adding ATP when the reaction rate, measured by ΔA or ΔA, slowed down, usually after approximately 2 h. When the reac-

Preliminary experiments demonstrated a disagreement between actual yields and expected yields based on spectrophotometric data. To investigate if this was due to oxidative formation of dimers, experiments were conducted where caffeoyl-CoA was synthesized under aerobic and anaerobic conditions. Table 1 shows recovered caffeoyl-CoA and caffeic acid. In the aerobic experiment much less of the total caffeic acid could be accounted for, but nevertheless the amounts of recovered thioester in the two experiments were the same. Since the yield of caffeoyl-CoA was the same in the two experiments it was decided to perform subsequent experiments under aerobic conditions, since this facilitates the experimental work considerably.

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Thioesterase Activity

Feruloyl-CoA, 30 mM, was incubated with 10 μl of enzyme preparation in a solution containing 0.2 M Mops (pH 7.5), 10 mM MgCl₂, in a total volume of 150 μl. The reaction took place overnight at room temperature. In a control incubation, water was added instead of enzyme.

TLC for SAM Analysis

Thin layer chromatography of SAM was made with a solvent system of 1 M ammonium acetate/ethanol (3:7 v/v).

RESULTS

An enzyme preparation from wheat was used to synthesize p-coumaryl-CoA, caffeoyl-CoA, and feruloyl-CoA. The enzyme preparation was very active in both p-coumaryl-CoA ligase, caffeoyl-CoA 3-O-methyltransferase and caffeic acid 3-O-methyltransferase. The ligase reaction was continuously monitored spectrophotometrically. This was practical in order to determine when to add ATP, phosphocreatine, creatine phosphokinase, and myokinase.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Recovery of Cinnamic Acids under Aerobic and Anaerobic Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔA₃₄₆</td>
</tr>
<tr>
<td>Aerobic</td>
<td>0.525</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>1.17</td>
</tr>
</tbody>
</table>

Note. The conversion of caffeic acid into caffeoyl-CoA under aerobic and anaerobic conditions was investigated in reactions with 400 nmol caffeic acid as substrate. The final amount of caffeic acid and caffeoyl-CoA was determined by HPLC.

FIG. 1. Time course for the formation of feruloyl-CoA using enzyme prepared from 2-day-old wheat seedlings. Amounts of feruloyl-CoA determined by HPLC (see text). The reaction contained 0.2 mM caffeic acid and 4 mM SAM. Additions at different time points. (1) ATP, (2) ATP, phosphocreatine, creatine phosphokinase, and myokinase.
tion slowed down again, usually after additional 2 h, ATP, phosphocreatine, creatine phosphokinase, and myokinase were added. Further additions did not increase the yield. Adding phosphocreatine, creatine phosphokinase, and myokinase from the start of the reaction did not result in as high yields and led to problems with precipitation. Excepting ATP at the second addition resulted in a very slow reaction rate, while omitting phosphocreatine and creatine phosphokinase at this point meant that almost no further conversion could be achieved. Myokinase had the effect of increasing the reaction rate but did not alter the final yields when added together with phosphocreatine and creatine phosphokinase. This suggests that the reaction is inhibited by AMP formed in the ligase reaction (23). The fact that ATP could not be omitted at this point is probably due to a large amount of ATP consuming enzymes in the preparation other than caffeoyl-CoA ligase. Increase in reaction rate when adding myokinase indicates that some endogenous myokinase was present in the enzyme preparation, but in limiting amounts.

Reactions that use SAM as a substrate are usually inhibited by S-adenosyl-L-homocysteine, and such reactions are driven by the presence of S-adenosyl-L-homocysteine hydrolase. Therefore, the in vitro methylation of caffeic acid and caffeoyl-CoA into the corresponding ferulic acid and feruloyl-CoA could be inhibited by S-adenosyl-L-homocysteine. To investigate this, S-adenosyl-L-homocysteine hydrolase was added after the maximum conversion into thioesters had been achieved. This did not lead to increased ratios of feruloyl-CoA to caffeoyl-CoA (data not shown), possibly because the enzyme preparation contained endogenous S-adenosyl-L-homocysteine hydrolase.

Optimal Age of Wheat Seedlings

In order to determine the optimal plant material, enzymes were prepared from wheat seedlings 2 to 9 days old. The enzyme preparations were used in reactions with caffeic acid, CoA, and SAM as described above and the yields of caffeoyl-CoA, feruloyl-CoA, and ferulic acid were determined by HPLC.

Two-day-old plants resulted in the highest yields of feruloyl-CoA, caffeoyl-CoA, and ferulic acid and were therefore chosen as material for further investigations (Fig. 2). At day 5 an increased yield of ferulic acid was observed, and since this took place without an increase in the ratio of feruloyl-CoA to caffeoyl-CoA it can be speculated that feruloyl-CoA was predominantly synthesized with caffeoyl-CoA as intermediate rather than by the action of the CoA ligase on ferulic acid. The presence of thioesterase activity (see below) does, however, make it difficult to make conclusions on the bio-

FIG. 2. Formation of feruloyl-CoA, caffeoyl-CoA, and ferulic acid when incubating 0.2 mM caffeic acid and 50 μM SAM with differently aged wheat seedlings. Caffeoyl- and feruloyl-CoA were determined by HPLC. ● Ferulic acid. ■ Feruloyl-CoA. ○ Caffeoyl-CoA.

synthetic pathways, since its specificity will to some extent determine the final products.

Stability of Feruloyl-CoA

When isolated feruloyl-CoA was incubated with the enzyme preparation used for synthesis, no feruloyl-CoA could be detected after 24 h. From a control incubated without enzyme, 65% of the feruloyl-CoA could be recovered. This demonstrated the presence of a thioesterase in the enzyme extract. This enzyme was still active after 5 h incubation as seen in Fig. 1, where the absorbance decreased after 5 h, the point of maximum conversion.

Optimal Concentration of SAM

When using nonradioactive substrate a maximal absolute yield of feruloyl-CoA is desirable. However, when using expensive 14C-labeled SAM, it is also important to consider the yield of feruloyl-CoA relative to the amount of SAM used. To determine the optimal conditions for producing nonradioactive and radioactive feruloyl-CoA, the reaction was carried out using different concentrations of SAM (Fig. 3). Not surprisingly the absolute yield of feruloyl-CoA was highest at high concentrations of SAM, a concentration of 0.5 mM was normally used. However, at this concentration only 3% of the methyl-groups from SAM were incorporated into feruloyl-CoA (Fig. 3). Lowering the SAM concentration below 75 μM resulted in incorporation of approximately 10% SAM methyl groups into feruloyl-CoA. The total yield of incorporated SAM could, however, be increased when lowering the concentration further. This was caused mostly by incorporation into ferulic acid. To ensure a high yield and a reasonable
Purification of Hydroxy Cinnamic Acids and Hydroxy Cinnamoyl-CoA Thioesters

The thioesters caffeoyl-CoA, p-coumaroyl-CoA, and feruloyl-CoA could be separated by reverse phase HPLC applying a solvent system of phosphoric acid and acetonitrile. They had the retention times of 32.4, 37.4, and 39.2 min, respectively, whereas the corresponding acids eluted at 23.4, 27.2, and 33.5 min.

Retention times of ferulic acid and caffeoyl-CoA are similar and since large amounts of caffeic acid and caffeoyl-CoA were present when making 14C-labeled feruloyl-CoA, it was found advantageous to extract the reaction mixture with ethylacetate prior to HPLC. This extraction of cinnamic acids is quantitative, as can be seen from the fact that no caffeic acid peak could be detected in the aqueous phase after the extraction (Fig. 4).

Figure 4 shows the resulting HPLC chromatograms when preparing 14C-labeled feruloyl-CoA under standard conditions. The caffeoyl-CoA peak had a large shoulder. Rechromatography of this peak resulted in two poorly separated peaks, both with the characteristic spectrum of caffeoyl-CoA. The most likely explanation for this is that both (Z) and (E) isomers of caffeoyl-CoA were present, although the substrate, caffeic acid, was predominantly the trans-isomer.

For making thioesters of high purity in dry state it was necessary to carry out one more purification step using a volatile solvent system (TFA, acetonitrile, methanol, water). In this solvent system, feruloyl-CoA eluted at 14.4 min, p-coumaroyl-CoA eluted at 13.9 min, and caffeoyl-CoA eluted as a single peak at 10.5 min. With this solvent system the collected peaks could be lyophilized without losses. Losses when rechromatographing the thioesters were a few percent for p-coumaroyl-CoA and feruloyl-CoA and 20% for caffeoyl-CoA. No explanation could be found for the larger loss when rechromatographing caffeoyl-CoA compared to the two other thioesters. The volatile solvent system could not be applied for routine purification since the products did not elute in as good purity as in the phosphoric acid–acetonitrile system. Furthermore, part of the product was easily lost due to overloading of the column when using the volatile solvent system in the first HPLC purification step.

Experiments with 14C-labeled SAM were used for estimating the loss in purification. After HPLC purification, 65–70% of the counts were found in the aqueous phase and 10–15% in the organic phase. In total 75–85% of the radioactivity could be accounted for. In Fig. 4 the radioactivity in the aqueous phase was separated.
into two groups of peaks, one group eluting at 2–14 min and the product eluting at 36–40 min. TLC of a SAM standard showed an Rf of 0.31 and analysis of the 2- to 14-min fractions showed that only the 2- to 4-min fractions contained intact SAM, whereas following fractions, from 4 to 14 min, were degradation products of SAM (data not shown). As shown in Figs. 3 and 4 ferulic acid was synthesized in significant amounts. Labeled ferulic acid could be purified and used in later incubations, so this side reaction did not lead to any additional loss. The purified thioesters could all be hydrolyzed to their corresponding acid and the characteristic spectra of thioesters could be recorded corresponding to the spectra published by Stöckigt and Zenk (16).

### Substrate Specificity of Caffeoyl:CoA Ligase

Three different hydroxy cinnamic acids were used: p-coumaric acid, caffeic acid, and ferulic acid. The reactions contained 0.2 mM of the appropriate acid, and CoA and ATP according to the standard conditions. ATP, phosphocreatine, creatine phosphokinase, and myokinase were added during the reaction as discussed above. The final yield of thioester was determined by HPLC. The initial reaction rate was determined from the absorbance change at 346 nm (caffeoyl-CoA and feruloyl-CoA) and 333 nm (p-coumaroyl-CoA). Final conversion into thioesters were the same for the three cinnamic acids (Table 2).

The much higher initial rate measured for p-coumaric acid may be an artifact, since this was measured at 333 nm, which is closer to the wavelengths where the cinnamic acids and their dimers absorb (24) than the 346 nm used for caffeoyl-CoA and feruloyl-CoA. Based on spectrophotometric measurements, approximately 35% of the hydroxy cinnamic acids are converted into thioesters, which is in good agreement with the figure reported by Meng and Campbell (20), also based on spectrophotometry. However as discussed above, spectrophotometric determination tends to overestimate the actual product formation due to oxidative side reactions.

### CONCLUSION

Feruloyl-CoA, p-coumaroyl-CoA, and caffeoyl-CoA are not commercially available. However, the procedure reported here is an efficient method for synthesizing these thioesters, which are important substrates for investigating the biosynthesis of lignin and esterified wall polysaccharides. The yields obtained are similar to those reported by Meng and Campbell using developing secondary xylem tissue from aspen (17). However, an advantage of the present system is the readily available enzyme source, since wheat seedlings can be easily grown at any time.

The data reported here also shows that the p-coumaric acid:CoA ligase and the caffeic acid and caffeoyl-CoA O-methyl transferases can be simultaneously active under in vitro conditions. Thus, it is possible to produce [14C]-feruloyl-CoA in a single reaction. Both the labeled and the unlabeled thioesters can be purified efficiently and with very little loss by the presented HPLC procedure.

The experiments reported here were carried out in relatively small scale (2 ml). However, the procedure can easily be scaled up, since it is possible to lyophilize the solutions with only minor losses prior to HPLC purification, which is the limiting step. The HPLC column used has the capacity to separate μmol amounts of cinnamic acid thioesters.

The feruloyl and p-coumaroyl transferases involved in the esterification of wall polysaccharides have not been studied in any detail, let alone identified. The procedure reported here should facilitate progress in the investigation of these enzymes.

### ACKNOWLEDGMENTS

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### REFERENCES