Enzymatic Synthesis of Phenolic CoAs Using 4-Coumarate:coenzyme A Ligase (4CL) from Rice

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Generally, biological synthesis using enzymes or cells has an advantage over chemical synthesis for compounds requiring chirality and regioselectivity.[1,2] In addition, reaction condition of biological synthesis is much milder and several synthetic steps can be omitted.[3,4] Thus, recently, many researches have been done to develop suitable biocatalysis.

During *in vitro* study on biosynthetic pathway, one of the difficulties was to find suitable substrates. For *in vitro* characterization of enzyme, special forms of intermediates are required, which need complicate reaction scheme for organic synthesis.[5] Among them, coenzyme A (CoA) thioesters are important intermediates found in many biological processes such as fatty acid metabolism, and citric acid cycle.[6] In the phenylpropanoid pathway leading to flavonoid, anthocyanin and lignin biosynthesis, several phenolic CoA thioesters serve as important substrates.[7]

The enzyme, 4-coumarate:CoA ligase (4CL) catalyzes the conversion of 4-coumaric acid into coumaroyl-CoA and a few related substrates into their corresponding products such as cinnamoyl-CoA, caffeoyl-CoA, and feruloyl-CoA in a process utilizing ATP and thus channels the common, phenylalanine-derived building block into the widely distinct branches of general phenylpropanoid metabolism.[8] The phenylpropanoid branch pathways have diverse functional compounds in plant development and environmental interactions, such as lignin for structure support, flavonoids for UV protection, and anthocyanins and chalcones as pigments for the attraction of pollinators. Furthermore, CoA esters made by 4-coumarate:CoA ligase (4CL) represent a key class of activated intermediates in these phenylpropanoid branch pathways.[9]

Currently, we have studied in phenylpropanoid and flavonoid biosynthesis in rice.[10,11] *In vitro* characterization of enzymes in these pathways has been hindered by the lack of intermediates such of caffeoyl-CoA. However, conventional chemical synthesis of these intermediate compounds required multiple synthetic steps with a low yield, whereas enzymatic synthesis requires just a single step. Thus, we searched for sequences highly homologous to 4CL from rice (R4CL). Two primers, 5'-GAGATTATGGGGTCGGTGG-3' as a forward primer and 5'-TTTTTCACCGGTTGTGTGAC-3' as a reverse primer were synthesized based on the R4CL sequence (GenBank Accession number BAD27987). *R4CL* was cloned by polymerase chain reaction with cDNA as a template and the above two primers. cDNA was synthesized with total RNA isolated from 3 weeks old rice seedling using omniscript reverse transcriptase (Qiagen, German). PCR was carried out with Hotstart Taq polymerase (Qiagen) under the following conditions: 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 2 min amplification at 72 °C. Then, PCR product was subcloned into pGEMT-Easy vector (Promega, USA) and its both strands were sequenced. The open reading frame of *R4CL* consisted of 1665 bp, which is predicted to encode 59.6-kDa protein.

In order to use R4CL for the attachment of CoA to phenolic compounds, the open reading frame of R4CL was subcloned into *Escherichia coli* expression vector pGEX 5X-1, which contains glutathione *S*-transferase. The resulting construct was transformed into *E. coli* BL21. Subsequently, *E. coli* harboring *R4CL* cultivated in LB medium containing 50 μg/mL ampicilin, and the culture was allowed to grow until absorbance at 600 nm reached 0.6 to 0.8. At this point, IPTG was added at a final concentration of 0.1 mM and the culture was incubated for additional 5 hours at 28 °C. The resulting recombinant protein was purified using glutathione *S*-transferase affinity column. The purified proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis. As shown in Figure 1, R4CL was successfully expressed in *E. coli* and purified to near homogeneity. The R4CL was purified by 10 fold with 8.4% yield. The molecular weight of the recombinant R4CL was about 86-kDa which corresponds well with the sum of the predicted molecular weight

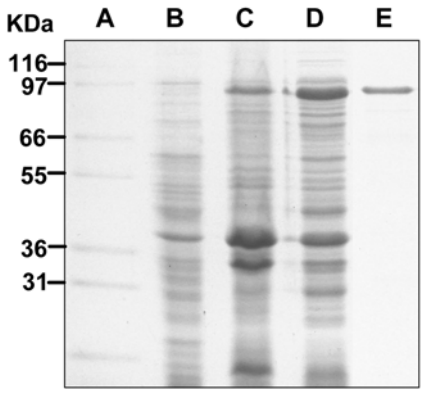


Figure 1. SDS-PAGE analysis of the recombinant R4CL. A, Molecular weight size markers; B, *E. coli* lysate before induction; C, *E. coli* lysate after induction; D, Soluble protein after sonication; E, purified 4CL with GST affinity column.

of R4CL (59.6-kDa) and the molecular weight of GST (26 kDa).

Enzymatic synthesis of phenolic CoA was carried out with the purified R4CL. The reaction mixture contained 100 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 2.5 mM ATP, 100 μM coenzyme A and 100 μM substrates (caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid) in 1 mL and the reaction was performed at room temperature. The reaction was stopped by 3.5% trichloroacetic acid in 50% acetonitrile and the reaction products were analyzed using Varian high performance liquid chromatography (HPLC; Palo Alto, CA, USA) equipped with C18 reversed-phase column (Waters Milford, MA, 4.60 × 250 mm, 0.6 micron) and a photo diode array detector. For analytical scale, the mobile phase consisted of 0.1% formic acid buffer (pH 3.0) was programmed as follow; 10% acetonitrile at 0 min, 40% acetonitrile at 10 min, 70% acetonitrile at 20 min, and 90% acetonitrile at 30 min. The flow rate was 1 mL/min and UV detection was performed at 340 nm. Analysis of reaction products with HPLC showed that caffeic acid, *p*-coumaric acid, and ferulic acid gave new peaks that had different retention time with substrate itself but analysis of sinapic acid reaction product did not show any new peaks. This result indicted that R4CL used caffeic acid, *p*-coumaric acid, and ferulic acid as a substrate. Furthermore, liquid chromatography mass spectrometry (LC-MS) was performed using a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Co., Madison, WI, USA) equipped with an electrospray ionization (ESI) source. HPLC separations was performed on the Finnigan Surveyor™ Modular HPLC Systems (Thermo Electron Co.) using a YMC Hydrosphere C18 (5 μm, 50 × 2.0 mm, YMC Co, Kyoto, Japan) with BetaBasic-18 guard column (2.1 × 10 mm, Thermo, USA). The system was operated by the Lee *et al.* method.[12]

Molecular weight of caffeic acid reaction product was *m/z* 930 ($[M+H]^+$) based on mass spectrometer, which agreed with the predicted molecular weight (MW 929) of the predicted caffeoyl-CoA (Fig. 2). Also, molecular weight of ferulic acid (*m/z* 944, $[M+H]^+$) and *p*-coumaric acid reaction product (*m/z* 914, $[M+H]^+$) agreed well with the predicted molecular weight of the predicted corresponding CoAs. Furthermore, relative reactivity of caffeic acid, *p*-coumaric acid, and ferulic acid was measured. Among them, R4CL exhibited the highest activities toward *p*-coumaric acid as a substrate. After 12 hrs incubation of 100 μM of caffeic acid, *p*-coumaric acid and ferulic acid with 50 μg of the purified R4CL at 25 °C, 62 μM of caffeoyl-CoA, 45 μM of feruloyl-CoA and 66 μM of *p*-coumaroyl-CoA could be obtained (Table 1).

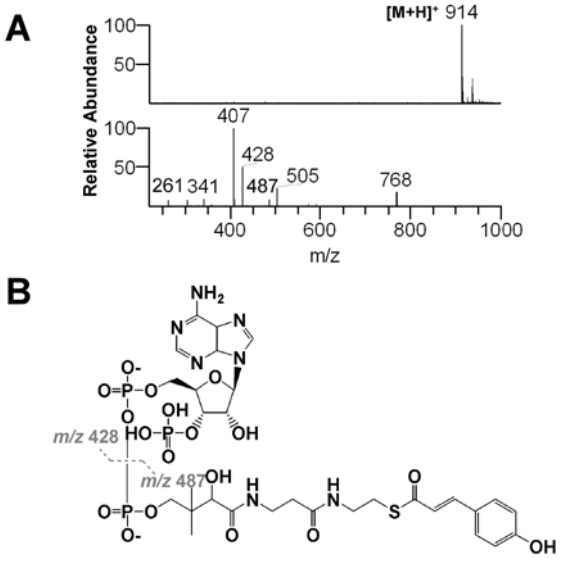
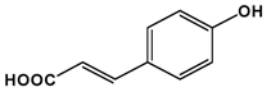
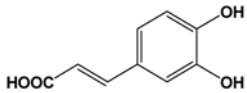
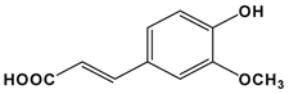


Figure 2. Mass spectrum and MS/MS fragment analysis of the *p*-coumaric acid reaction product with R4CL2. A, Mass and MS/MS spectrum of the *p*-coumaric acid reaction product; B, identified fragments of *p*-coumaroyl CoA by MS/MS analysis.

Table 1. Conversion of R4CL with *p*-coumaric acid, caffeic acid, and ferulic acid

Substrate	Structure	Yield (μM)
p-Coumaric acid		66
Caffeic acid		62
Ferulic acid		45

Sinapic acid was not served as a substrate for R4CL. Fifty μg of the purified R4CL was used with 100 μM of each substrate. Reaction mixture was incubated at 25 °C for 12 hrs.

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