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Identification and characterization of multiple curcumin synthases from the herb *Curcuma longa*

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ABSTRACT

Curcuminoids are pharmaceutically important compounds isolated from the herb *Curcuma longa*. Two additional type III polyketide synthases, named CURS2 and CURS3, that are capable of curcuminoid synthesis were identified and characterized. In vitro analysis revealed that CURS2 preferred feruloyl-CoA as a starter substrate and CURS3 preferred both feruloyl-CoA and *p*-coumaroyl-CoA. These results suggested that CURS2 synthesizes curcumin or demethoxycurcumin and CURS3 synthesizes curcumin, bisdemethoxycurcumin and demethoxycurcumin. The availability of the substrates and the expression levels of the three different enzymes capable of curcuminoid synthesis with different substrate specificities might influence the composition of curcuminoids in the turmeric and in different cultivars.

1. Introduction

Curcuminoids, natural products isolated from the rhizome of turmeric (*Curcuma longa*), have long been used as a traditional Asian medicine and food additives [1]. Curcuminoids possess various biological activities beneficial to human health, including anti-inflammatory, antioxidant, and antitumor activities and activity to decrease the amyloid pathology of Alzheimer's disease [1,2]. Therefore, the biosynthesis of curcuminoids has attracted much interest and has been studied by many researchers since decades ago [3–6]. The rhizome of turmeric contains a mixture of curcuminoids, mainly including curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**).

Type III polyketide synthases (PKSs), consisting of a homodimer of ketosynthase, are structurally simple enzymes and involved in the biosynthesis of most plant polyketides [7]. A typical type III PKS catalyzes condensation of a carboxylic acid coenzyme A (CoA) ester, which is called a starter substrate, with several molecules of malonyl-CoA, which is called an extender substrate, and subsequent cyclization of the resultant polyketide chain [7]. We have recently found that curcuminoids in the herb *C. longa* are synthesized by a collaboration of two type III PKSs, diketide-CoA synthase (DCS) and curcumin synthase 1 (CURS1, the first identified CURS) (Fig. 1A) [8]. DCS catalyzes formation of feruloyldiketide-CoA (**4**) from feruloyl-CoA (**5**) and malonyl-CoA. CURS1 catalyzes formation of curcumin from feruloyl-CoA (**5**) and the feruloyldiketide-CoA produced by the action of DCS (**4**). Thus, DCS and CURS1 catalyze the formation of curcumin. Both enzymes accept *p*-coumaroyl-CoA (**6**), but at low efficiency, and are also capable of synthesizing bisdemethoxycurcumin (**3**) from *p*-coumaroyl-CoA (**6**) and malonyl-CoA via *p*-coumaroyldiketide-CoA (**7**) formation. Although a pair of DCS and CURS produces a mixture of curcuminoids; i.e., curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin, from feruloyl-CoA (**5**), *p*-coumaroyl-CoA (**6**) and malonyl-CoA in vitro, it yields the mixture of products with a composition different from that of an ethyl acetate extract of the rhizome of turmeric; the rhizome of turmeric contains a relatively larger amount of bisdemethoxycurcumin (**3**) than the in vitro reaction products by a pair of DCS and CURS. Therefore, we assumed that the composition of curcuminoids in the mixture might be regulated by the concentrations of *p*-coumaroyl-CoA and feruloyl-CoA in vivo [8].

In the present study, we cloned and characterized two novel type III PKSs from turmeric. These two type III PKSs, named CURS2 and CURS3, showed CURS-like activity with the substrate

Abbreviations: CHS, chalcone synthase; CoA, coenzyme A; CURS, curcumin synthase; DCS, diketide-CoA synthase; HPLC, high-performance liquid chromatography; LC-APCIMS, liquid chromatography-atmospheric pressure chemical ionization mass spectrometry; NAC, *N*-acetylcysteamine; PKS, polyketide synthase

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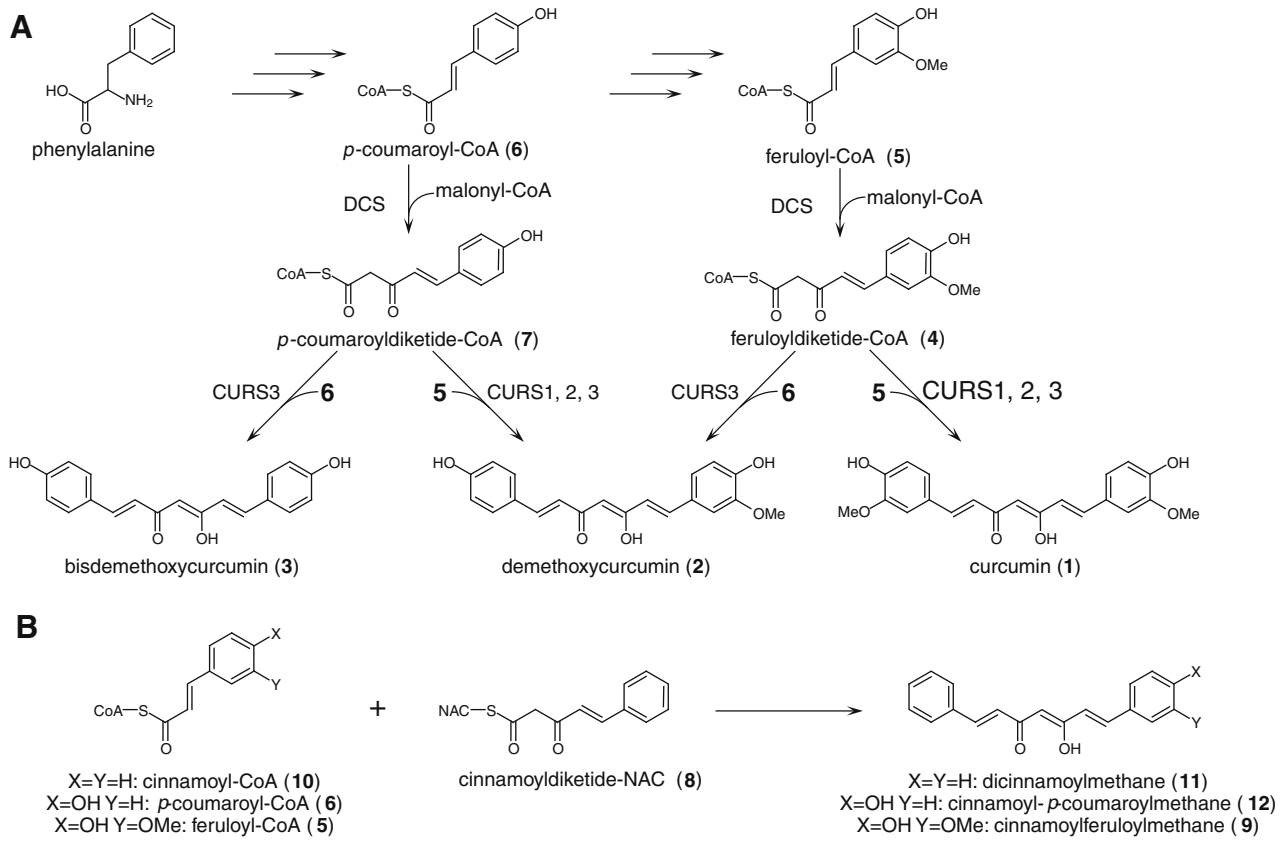

Fig. 1. The biosynthesis pathway of curcuminoids (A) and the reactions catalyzed by curcumin synthases (CURSs) (B). *A*, Feruloyl-coenzyme A (CoA) (**5**) synthesized from phenylalanine are condensed with malonyl-CoA and convereted to feruloyldiketide-CoA (**4**) by diketide-CoA synthase (DCS). The synthesized feruloyldiketide-CoA (**4**) is condensed with feruloyl-CoA (**5**) by the actions of CURS1, 2, and 3 and condensed with *p*-coumaroyl-CoA (**6**) by the action of CURS3 to yield curcumin (**1**) and demethoxycurcumin (**2**). When *p*-coumaroyl-CoA (**6**) is used by DCS, *p*-coumaroyldiketide-CoA (**7**) is produced and demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**) are produced by CURSs in a similar manner. CURS1 and CURS2 prefer feruloyl-CoA (**5**) and CURS3 accepts both feruloyl-CoA (**5**) and *p*-coumaroyl-CoA (**6**). *B*, CURSs catalyze the formation of curcuminoids (**1**–**3**) from cinnamoyl-CoA (**10**), *p*-coumaroyl-CoA (**6**) and feruloyl-CoA (**5**), when incubated with cinnamoyldiketide-*N*-acetylcysteamine (NAC) (**8**), an analogue of diketide-CoA.

specificity slightly different from that of CURS1 and synthesized curcumin (**1**) from feruloyldiketide-CoA (**4**) and feruloyl-CoA (**5**) in vitro. The presence of multiple type III PKSs responsible for curcuminoid synthesis in the turmeric of *C. longa* suggests that the expression levels of *CURS*, *CURS2* and *CURS3* are also important for determining the composition of the curcuminoid mixture, in addition to the availability of the substrates, *p*-coumaroyl-CoA and feruloyl-CoA.

2. Materials and methods

2.1. Materials

Escherichia coli JM109, plasmid pUC19, restriction enzymes, T4 DNA ligase, and *Taq* DNA polymerase were purchased from Takara Biochemicals (Shiga, Japan). pET16b was used for expression of His-tagged proteins in *E. coli* BL21 (DE3) (Novagen; Darmstadt, Germany). Bisdemethoxycurcumin (**3**) was purchased from Chromadex (Santa Ana, CA). Curcumin (**1**) was purchased from Sigma (Steinheim, Germany). *trans*-Cinnamic acid, *p*-coumaric acid, and ferulic acid were purchased from Wako (Tokyo, Japan). *p*-Coumaroyl-CoA (**6**), cinnamoyl-CoA (**10**), and feruloyl-CoA (**5**) were synthesized according to the procedures reported by Blecher [9]. Cinnamoyldiketide-*N*-acetylcysteamine (NAC), cinnamoyl-*p*-coumaroylmethane (**12**), dicinnamoylmethane (**11**), and cinnamoylferuloylmethane (**9**) were synthesized according to the previously reported method [10,11].

2.2. Amplification of full-length cDNAs

A cDNA library was prepared as described previously [8]. The full-length cDNA of *CURS2* was amplified by PCR using a pair of primers, 5′-GCTAATCAGTCAATCCAGATGG-3′ and 5′-CGTCTATCGATTGATCGATCGTG-3′, and the cDNA library as a template. These primers were designed on the basis of the previously reported expressed sequence tag (EST) sequences (NCBI accession no. DY393763 and DY387045). A 1.3-kb cDNA fragment encoding *CURS2* was obtained.

The full-length cDNA of *CURS3* was amplified by carrying out 3′-rapid amplification of cDNA ends using a primer 5′-CTGCTAGCTAGCTGCAATTCG-3′ and a SMART RACE cDNA amplification kit (Clontech). The primer was also designed on the basis of the previously reported EST sequences (NCBI accession no. DY394591). The amplified 3′-RACE fragment was purified and sequenced, yielding a 1.3-kb cDNA fragment encoding *CURS3*.

2.3. Relative quantification of transcription by quantitative real time PCR

Quantitative real time PCR was carried out as described previously using gene-specific forward and reverse primers (5′-TGTTGCCGAACTCGGAGAAGAC-3′ and 5′-TCGGGATCAAGGACTGGAACAAC-3′ for CURS2; 5′-CCCATTCCTTGATCGCCTTTTCC-3′ and 5′-TGGAGCCCTCCTTCGACGACC-3′ for CURS3; and 5′-CCTTCCTCTAAATGATAAGGTTCAATGG-3′ and 5′- GATTGAATGGTCCGGTGAAGTGTT-3′ for 18S rRNA) [8].

2.4. Production and purification of CURS2 and CURS3

Using the *CURS2* cDNA as a template, a 1.2-kb DNA fragment containing the CURS2-coding region was amplified by PCR with the primers 5′-CC**GAATTC**CA*TATG*GCGATGATCAGCTTGCA-3′ (the bold letters indicate an EcoRI site, the underline indicates an NdeI site, and the italics indicate the start codon of *CURS2*) and 5′-CGC**GGATCC***CTA*AAGCGGCACGCTTTGGA-3′ (the bold letters indicate a BamHI site and italics indicate the stop codon of *CURS2*). The amplified fragment was cloned between the EcoRI and BamHI sites of pUC19, resulting in pUC19-CURS2. The *CURS2* sequence was excised as an NdeI–BamHI fragment from pUC19-CURS2 and cloned between the NdeI and BamHI sites of pET16b, resulting in pET16b-CURS2. His-tagged CURS2 was produced and purified as for purification of CURS [8].

Using the *CURS3* cDNA as a template, a 1.2-kb DNA fragment containing the CURS-coding region was amplified by PCR with the primers 5′-CC**GAATTC**CA*TATG*GGCAGCCTGCAGGCGAT-3′ (the bold letters indicate an EcoRI site, the underline indicates an NdeI site, and the italics indicate the start codon of *CURS3*) and 5′-CGC**GGATCC***CTA*CGGTAATGGTACACTGC-3′ (the bold letters indicate a BamHI site and italics indicate the stop codon of *CURS3*). The amplified fragment was cloned between the EcoRI and BamHI sites of pUC19, resulting in pUC19-CURS3. The *CURS3* sequence was excised as an NdeI–BamHI fragment from pUC19-CURS3 and cloned between the NdeI and BamHI sites of pET16b, resulting in pET16b-CURS3. His-tagged CURS3 was similarly produced and purified as for CURS2.

2.5. CURS assay

The standard reaction mixture contained 100 μM starter substrate [cinnamoyl-CoA (**10**), *p*-coumaroyl-CoA (**6**), or feruloyl-CoA (**5**)], 100 μM extender substrate [malonyl-CoA or cinnamoyldiketide-NAC (**8**)], 100 mM potassium phosphate buffer (pH 7.5), and 4.0 μg CURS2 or CURS3 in a total volume of 100 μL. Reactions were incubated at 37 °C for 1 h before being quenched with 20 μL of 6 M HCl. The products were extracted with ethyl acetate, and the organic layer was evaporated to dryness. The residual material was dissolved in 20 μL of dimethylsulfoxide for liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCIMS/MS) analysis, which was carried out using the esquire high-capacity trap (HCT) system (Bruker Daltonics, Bremen, Germany). The high-performance liquid chromatography (HPLC) was equipped with a Pegasil-B C4 reversed-phase HPLC column (4.6 × 250 mm; Senshu, Tokyo) and eluted with a linear acetonitrile gradient (10–100% over 45 min) in water containing 0.1% acetic acid at a flow rate of 1.0 mL/min. UV spectra were detected on an Agilent G1315B system.

2.6. Determination of the kinetic parameters of CURSs

The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 1–100 μM each of feruloyl-CoA (**5**) or *p*-coumaroyl-CoA (**6**), 100 μM cinnamoyldiketide-NAC (**8**), and 4.0 μg of CURSs in a total volume of 100 μL. After the reaction mixture had been preincubated at 37 °C for 2 min, reactions were initiated by adding the substrates and continued for 5 min. The curcuminoid production was detected by following the absorbance at 420 nm. UV absorbance was detected using a Spectra MAX plus (Molecular Device; Sunnyvale, CA, USA). Cinnamoyl-*p*-coumaroylmethane (**12**) and cinnamoylferuloylmethane (**9**) were used to generate the standard curves for quantification of the products. Steady-state parameters were determined by using lineweaver–burk plot.

3. Results and discussion

3.1. Cloning of cDNAs for CURS2 and CURS3

Our search for possible type III PKS genes in the expressed sequence tag (EST) database of the herb *C. longa* in NCBI predicted that there were several genes probably encoding a type III PKS, in addition to DCS (BAH56225) and CURS (BAH56226), both of which were characterized as type III PKSs responsible for the synthesis of curcuminoids [8]. We tried to amplify these cDNAs encoding type III PKSs by using the primers based on the EST sequences (DY393763, DY387045, and DY394591). As a result, we succeeded in amplification of two cDNA fragments encoding probable type III PKSs. The gene obtained with the primers based on DY393763 and DY387045 was named CURS2 (AB506762), and that based on DY394591 was named CURS3 (AB506763). We confirmed that these genes were expressed in the rhizome and leaves of turmeric by quantitative PCR (Supplementary Fig. S1). As was observed for CURS1 [8], the expression level of *CURS2* in the rhizome was higher than in the leaves. In contrast, the expression levels of *CURS3* in the rhizome and leaves were similar. Therefore, CURS2 and CURS3 may exert different functions in the curcuminoid biosynthesis.

Both CURS2 and CURS3 possessed a Cys-His-Asn catalytic triad that is conserved in all known type III PKSs [12,13] (Supplementary Fig. S2). The amino acid identity between CURS2 and CURS3 was 81%. CURS2 shared 78%, 62% and 59% amino acid sequence identity with CURS1, DCS and the chalcone synthase (CHS) from *Medicago*

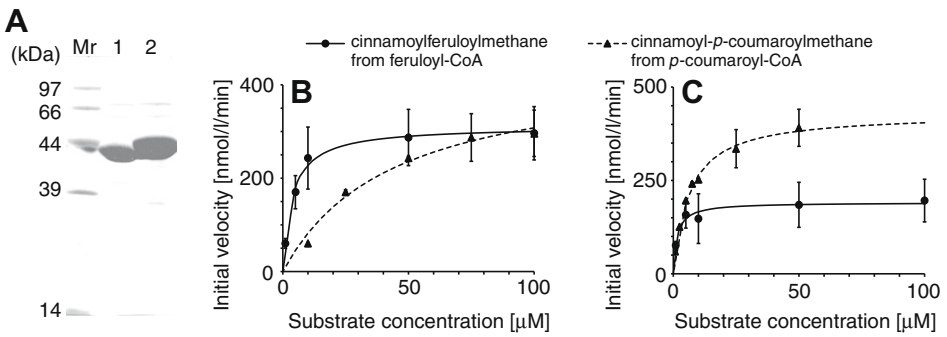

Fig. 2. Kinetic analysis of CURS2 and CURS3. SDS–polyacrylamide gel electrophoresis analysis of recombinant His-tagged CURS2 (lane 2) and CURS3 (lane 3) that were purified by nickel-nitrilotriacetic acid affinity column chromatography is shown (A). Lane Mr contains size markers. Initial velocity-substrate concentration profiles of CURS2 (B) and CURS3 (C) are presented. The kinetic parameters of CURS2 and CURS3 were measured by monitoring the formation of cinnamoylferuloylmethane and cinnamoyl-*p*-coumaroylmethane synthesized from feruloyl-CoA and *p*-coumaroyl-CoA, when incubated with cinnamoyldiketide-NAC, respectively. Kinetic curves for the CURSs incubated with feruloyl-CoA and *p*-coumaroyl-CoA are shown in filled circles and triangles.

sativa [12,13], respectively. CURS3 shared 81% and 66% and 58% amino acid sequence identity with CURS1, DCS and CHS from *M. sativa*, respectively.

3.2. Analysis of the reactions catalyzed by CURS2 and CURS3

Because of the high sequence identity of CURS2 and CURS3 with CURS1, we predicted that these enzymes might possess CURS-like activity. CURS2 and CURS3 fused with a His tag at their N termini were produced in *E. coli* BL21 (DE3) by using the pET system and purified by a nickel-nitrilotriacetic acid affinity column, giving a major protein band on SDS–polyacrylamide gel electrophoresis (Fig. 2A).

Incubation of the purified enzymes in the presence of feruloyl-CoA (**5**) and malonyl-CoA gave no detectable products (Fig. 3A and D). However, both CURS2 and CURS3 gave cinnamoylferuloylmethane (**9**) when incubated with feruloyl-CoA (**5**) and cinnamoyldiketide-NAC (**8**) (an analogue of feruloyldiketide-CoA) (Fig. 3B and E). In addition, a significant amount of curcumin (**1**) was produced from feruloyl-CoA (**5**) and malonyl-CoA when CURS2 and CURS3 were incubated together with DCS (Fig. 3C and F). CURS2 and CURS3 also produced bisdemethoxcurcumin when incubated with DCS, *p*-coumaroyl-CoA and malonyl-CoA (data not shown). Cinnamoylferuloylemthane (**9**), curcumin (**1**), and bisdemethoxycurcumin (**3**) were identified by comparing their retention times on HPLC and UV-, MS- and MS/MS-spectra with those of the authentic samples (Fig. 3G and H). All these data suggested that CURS2 and CURS3 were isozymes of CURS1.

The pH optima of CURS2 and CURS3 were near 8 and temperature optimum of CURS2 and CURS3 were near 45 °C and 55 °C, respectively (data not shown). We next examined the substrate specificity of theses enzymes by incubating in the presence of cinnamoyldiketide-NAC (**8**) and feruloyl-CoA (**5**), *p*-coumaroyl-CoA (**6**) or cinnamoyl-CoA (**10**). As a result, both enzymes produced only a trace amount of dicinnamoylmethane (**11**) from cinnamoyl-CoA (**10**) and cinnamoyldiketide-NAC (**8**). In contrast, CURS2 and CURS3 synthesized cinnamoyl-*p*-coumaroylmethane (**12**) and cinnamoylferuloylmethane (**9**) from *p*-coumaroyl-CoA (**6**) and feruloyl-CoA (**5**) when incubated with cinnamoyldiketide-NAC (**8**). The kinetic parameters of these enzymes for the cinnamoyl-*p*-coumaroylmethane (**12**) and cinnamoylferuloylmethane (**9**) syntheses activity from *p*-coumaroyl-CoA (**6**) and feruloyl-CoA (**5**), when incubated with cinnamoyldiketide-NAC, were determined to get an insight into substrate specificities of these enzymes (Table 1). The initial velocity-substrate concentration profiles of CURS2 and CURS3 are shown in Fig. 2B and C. As does CURS1 [8], CURS2 exhibited a larger K_{cat}/K_m value for feruloyl-CoA (**5**) than that for *p*-coumaroyl-CoA (**6**). Therefore, CURS2 seemed to prefer feruloyl-CoA (**5**). In contrast, the K_{cat}/K_m values of CURS3 for feruloyl-CoA (**5**) and *p*-coumaroyl-CoA (**6**) were similar. Therefore, CURS3 accepted *p*-coumaroyl-CoA (**6**) and feruloyl-CoA (**5**) at the same efficiency, showing substrate specificity slightly different from that of CURS1 and CURS2.

3.3. Multiple curcumin synthases in turmeric

The present study has demonstrated that the herb *C. longa* contains at least two type III PKSs, CURS2 and CURS3, that are capable of curcuminoid biosynthesis, in addition to the previously identified CURS1 [8]. CURS1 and CURS2 showed similar substrate specificity; they preferred feruloyl-CoA (**5**) as a starter substrate. On the other hand, CURS3 showed substrate specificity different from that of CURS1 and CURS2; it preferred both *p*-coumaroyl-CoA (**6**) and feruloyl-CoA (**5**) as a starter substrate. Although these characteristics were obtained from the kinetic analysis using cinnamoyldiketide-NAC, which is a mimic of the natural substrates, these results suggested that CURS2, like CURS1, mainly catalyzes formation of curcumin (**1**) and demethoxycurcumin (**2**) by condensing feruloyl-CoA with *p*-coumaroyldiketide-CoA (**7**) or feruloyldiketide-CoA (**4**) as an extender substrate (Fig. 1A). In contrast, CURS3 prob-

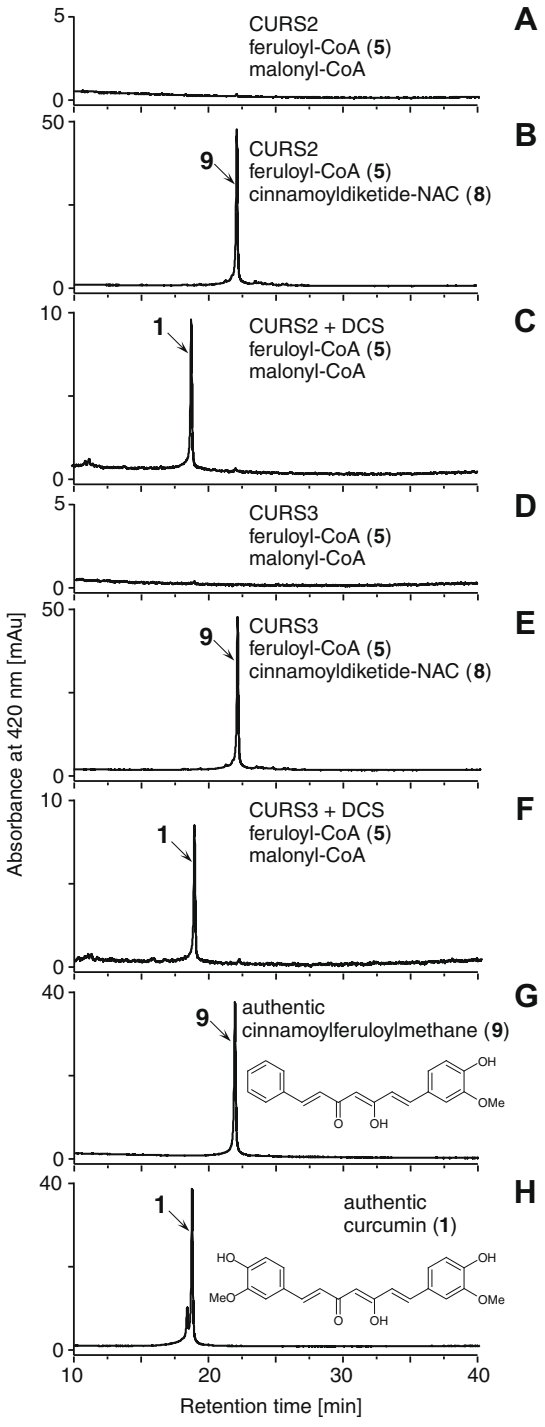

Fig. 3. High-performance liquid chromatography (HPLC) analysis of the reaction products of CURS2 and CURS3. Neither CURS2 (A) nor CURS3 (D) yielded detectable products, when incubated with malonyl-CoA and feruloyl-CoA (**5**). Incubation of CURS2 (B) and CURS3 (E) in the presence of feruloyl-CoA (**5**) and cinnamoyldiketide-NAC (**8**) resulted in the formation of cinnamoylferuloylmethane (**9**). CURS2 (C) and CURS3 (F), when incubated together with DCS, synthesized a significant amount of curcumin (**1**) from feruloyl-CoA (**5**) and malonyl-CoA. Cinnamoylferuloylmethane (**9**) (G) and curcumin (**1**) (H) were identified by comparing their retention times on HPLC and UV-, MS- and MS/MS-spectra with those of the authentic samples.

Table 1
Kinetic parameters of CURS2 and CURS3.

	CURS2		CURS3		CURS1	
	p-Coumaroyl-CoA	Feruloyl-CoA	*p*-Coumaroyl-CoA	Feruloyl-CoA	*p*-Coumaroyl-CoA	Feruloyl-CoA
K_m (μM)	89 ± 4	4.3 ± 1.0	3.4 ± 0.1	2.2 ± 1.1	189 ± 6	18 ± 5
K_{cat} (min^{-1})	0.94 ± 0.05	0.41 ± 0.11	0.36 ± 0.03	0.19 ± 0.03	0.85 ± 0.04	1.1 ± 0.4
K_{cat}/K_m (s^{-1} M^{-1})	176 ± 6	1622 ± 186	1742 ± 191	2017 ± 651	75 ± 0.4	1001 ± 96

Results are mean ± S.E. (n = 3). The data for CURS1 [8] are also presented for comparison.

ably catalyzes synthesis of three curcuminoids (**1**, **2** and **3**) by condensing feruloyl-CoA or *p*-coumaroyl-CoA with *p*-coumaroyldiketide-CoA (**7**) or feruloyldiketide-CoA (**4**) as an extender substrate (Fig. 1A). The rhizome of turmeric mainly contains three curcuminoids: curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**) [14]. The existence of three different type III PKSs in *C. longa* suggests that the composition of the curcuminoids in the rhizomes of turmeric is affected not only by the availability of the substrates, *p*-coumaroyl-CoA (**6**) and feruloyl-CoA (**5**) [8], but also by the expression levels of the genes encoding these enzymes. Furthermore, the observed difference in the composition of the curcuminoid compounds in different cultivars of turmeric [14,15] can also be accounted for by the two reasons.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.029.

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