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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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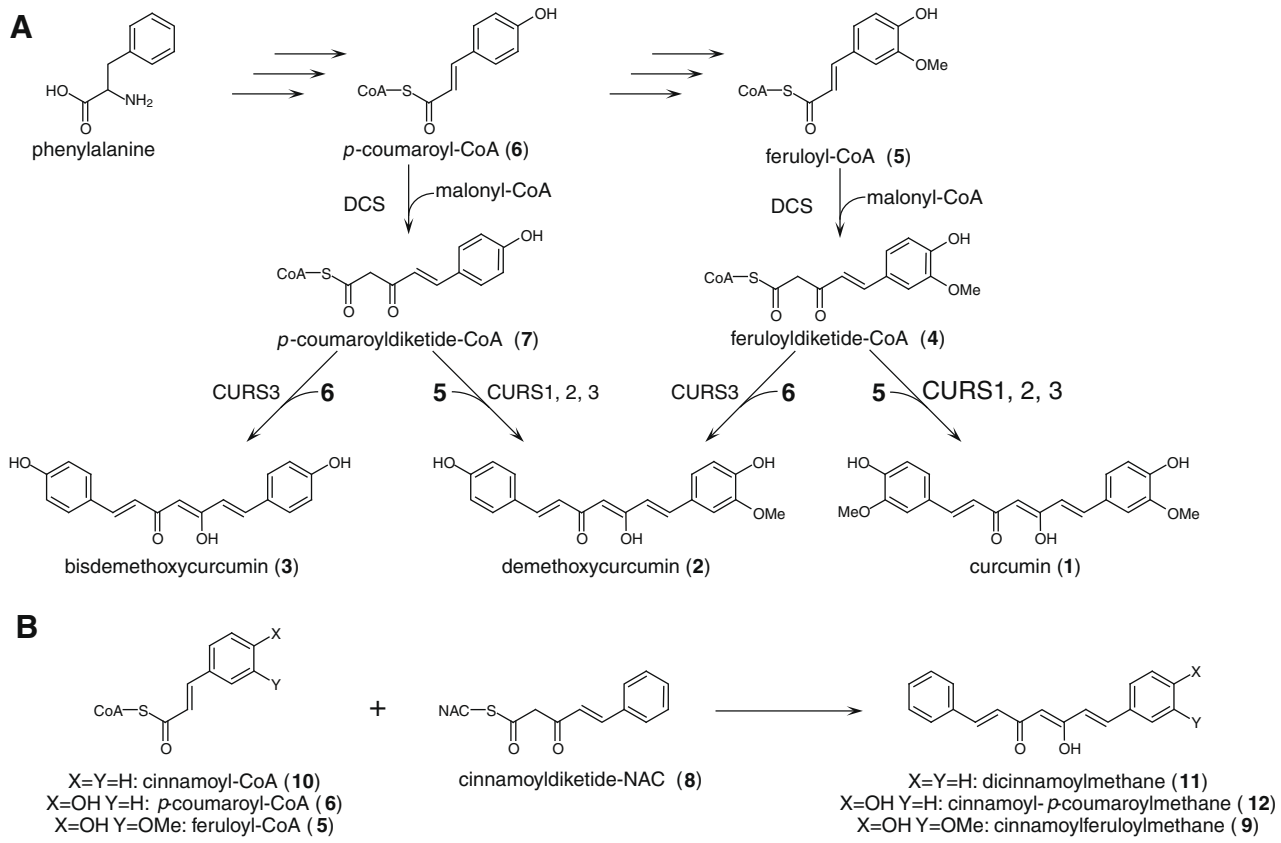

Fig. 1. The biosynthesis pathway of curcuminoids (A) and the reactions catalyzed by curcumin synthases (CURSs) (B). *A*, Feruloyl-coenzyme A (CoA) (**5**) synthesized from phenylalanine are condensed with malonyl-CoA and convereted to feruloyldiketide-CoA (**4**) by diketide-CoA synthase (DCS). The synthesized 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].