The first plant type III polyketide synthase that catalyzes formation of aromatic heptaketide

Ikuro Abe*, Yoriko Utsumi, Satoshi Oguro, Hiroshi Noguchi

School of Pharmaceutical Sciences, and the 21st Century COE Program, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

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Abstract A cDNA encoding a novel plant type III polyketide synthase (PKS) was cloned from rhubarb (*Rheum palmatum*). A recombinant enzyme expressed in *Escherichia coli* accepted acetyl-CoA as a starter, carried out six successive condensations with malonyl-CoA and subsequent cyclization to yield an aromatic heptaketide, aloesone. The enzyme shares 60% amino acid sequence identity with chalcone synthases (CHSs), and maintains almost identical CoA binding site and catalytic residues conserved in the CHS superfamily enzymes. Further, homology modeling predicted that the 43-kDa protein has the same overall fold as CHS. This provides new insights into the catalytic functions of type III PKSs, and suggests further involvement in the biosynthesis of plant polyketides.

Key words: Plant polyketide; Type III polyketide synthase; Chalcone synthase superfamily enzyme

1. Introduction

The chalcone synthase (CHS) (EC 2.3.1.74) superfamily of type III polyketide synthases (PKSs) are pivotal enzymes in the biosynthesis of flavonoids as well as a wide range of biologically important natural products [1,2]. They are structurally and mechanistically distinct from the type I (modular type) and type II (subunit type) PKSs, using free CoA thioesters as substrates without the involvement of acyl carrier protein; they typically select 4-coumaroyl-CoA as a starter and perform up to three condensations with malonyl-CoA [3]. Recently, a growing number of functionally diverse plant type III PKSs such as CHS, stilbene synthase (STS) [4], acridone synthase (ACS) [5], and 2-pyrone synthase (2PS) [6], sharing 60–75% amino acid sequence identity with each other, have been cloned and characterized. In addition, bacterial type III PKSs involved in the biosynthesis of a pentaketide 1,3,6,8-tetrahydroxynaphthalene have also been reported [7]. These CHS superfamily enzymes are homodimers of relatively modest-sized proteins of 40–45 kDa, catalyzing the assembly of complex natural products by successive decarboxylative condensations of malonyl-CoA in a biosynthetic process that closely parallels fatty acid biosynthesis. Thus, CHS performs sequential condensations of 4-coumaroyl-CoA with three C_2 units from malonyl-CoA followed by a Claisen-type cyclization reaction, leading to the formation of an aromatic tetraketide, naringenin chalcone (Fig. 1A). Crystallographic and site-directed mutagenesis studies on alfalfa (*Medicago sativa*) CHS revealed the active site machinery of the chalcone-forming reaction which proceeds through starter molecule loading at Cys164, malonyl-CoA decarboxylation, polyketide chain elongation, followed by cyclization and aromatization of the enzyme-bound tetraketide intermediate [8–15].

Rhubarb (*Rheum palmatum*, Polygonaceae) is a medicinal plant that produces a variety of aromatic polyketides including chromones, naphthalenes, anthraquinones, phenylbutanones, and stilbenes (Fig. 2) [16]. Therefore, in addition to regular CHSs involved in the biosynthesis of flavonoids, the presence of functionally different PKSs catalyzing the initial key reactions in the biosynthesis of these metabolites were expected. Indeed, we have previously reported cloning and characterization of benzalacetone synthase (BAS) (EC 2.3.1.-), a diketide synthase involved in the biosynthesis of lindleyin, the active principle of the anti-inflammatory action of the medicinal plant [16,17]. Here we describe the cloning and characterization of aloesone synthase (ALS) (EC 2.3.1.-), a novel CHS superfamily enzyme that plays a crucial role in the biosynthesis of chromones (Fig. 1B). This is the first plant-specific type III PKS catalyzing formation of an aromatic heptaketide, aloesone (2-acetonyl-7-hydroxy-5-methylchromone), from acetyl-CoA and six molecules of malonyl-CoA.

2. Materials and methods

2.1. Chemicals

[2-^{14}C]Malonyl-CoA (48 mCi/mmol) and [1-^{14}C]acetyl CoA (47 mCi/mmol) was purchased from Moravek Biochemicals (California, USA). Aloesone was isolated from crude aloin powder (Wako, Japan).

2.2. cDNA cloning

Young roots of *R. palmatum* were harvested at the medicinal plant garden of the University of Shizuoka in July 2000, and immediately frozen with liquid nitrogen. Total RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method, and reverse-transcribed using Reverscript (Wako) and oligo(dT) primer (rapid amplification of cDNA ends (RACE) 32 = 5′-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T-3′) according to the manufacturer's protocol. The obtained cDNA mixture was diluted with TE (10 mM Tris, 1 mM EDTA, pH 8.0) and used as a template for the following polymerase chain reactions (PCR). As described before [16], inosine-containing degenerate oligonucleotide primers based on the highly conserved sequences of known CHSs were used for amplification of a core 571-bp cDNA fragment; 112S = 5′-(A/G)A(A/G) GCI ITI (A/C)A(A/G) GA(A/G) TGG GGI CA-3′, 174S = 5′-GCI AA(A/G) GA(T/C) ITI GCI GA(A/G) AA(T/

*Corresponding author. Fax: (81)-54-264 5662.
E-mail address: abei@ys7.u-shizuoka-ken.ac.jp (I. Abe).

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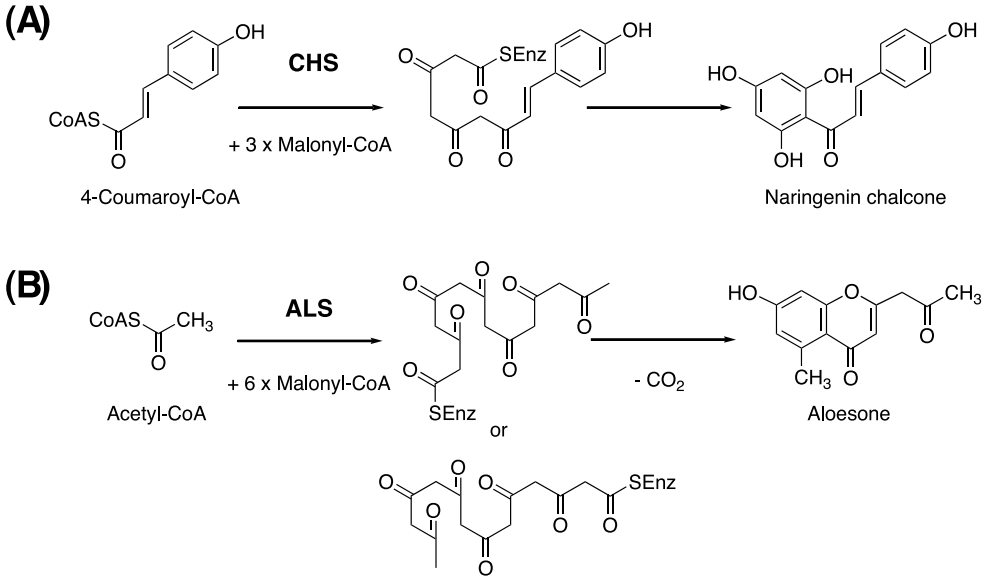

Fig. 1. Proposed mechanism for the conversion of (A) 4-coumaroyl-CoA and malonyl-CoA to naringenin chalcone by CHS, and (B) acetyl-CoA and malonyl-CoA to aloesone by ALS.

C) AA-3′, 368A = 5′-CCC (C/A)(A/T)I TCI A(A/G)I CCI TCI CCI GTI GT-3′, and 380A = 5′-TCI A(T/C)I GTI A(A/G)I CCI GGI CC(A/G) AA-3′ (the number of the primer indicates the amino acid number of the corresponding *M. sativa* CHS).

Then, 3′-RACE using two specific primers, 278S = 5′-GAC AAG ATC GAG GAG AAG C-3′ and 327S = 5′-GAT TCC CGC TAC ATC TTG-3′, amplified a 365-bp DNA fragment, while 5′-RACE was carried out using the Marathon™ cDNA amplification kit (Clontech) and two specific primers, 331A = 5′-AGA TGT AGC GGG TAT CCC GCA T-3′ and 232A = 5′-TCT CGT CGA TGT CTA TCC CGA-3′, to amplify a 842-bp DNA fragment.

2.3. Expression of cDNA

A full-length cDNA was obtained using N- and C-terminal PCR primers: 5′-GCG TGT CAT ATG GCA GAT GTC CTG CAG GAG-3′ (the *Nde*I site is underlined) and 5′-GAT GAG GTC GAC GGG AAT GGG GAC GCT ACG-3′ (the *Sal*I site is underlined). The amplified DNA was digested with *Nde*I/*Sal*I, and cloned into the *Nde*I/*Sal*I site of pET-22b(+) (Novagen). Thus, the recombinant enzyme contains an additional hexahistidine tag at the C-terminus. After confirmation of the sequence, the plasmid was transformed into *Escherichia coli* BL21(DE3)pLysS. The cells harboring the plasmid were cultured to A_{600} 0.6 in Luria–Bertani medium containing 100 μg/ml of ampicillin at 30°C. Then, 0.4 mM isopropyl thio-β-D-galactoside was added to induce protein expression, and the culture was incubated further at 16°C for 14 h.

2.4. Enzyme purification

The *E. coli* cells were harvested by centrifugation and resuspended in 40 mM potassium phosphate buffer (KPB), pH 7.9, containing 0.1 M NaCl. Cells were disrupted by sonication, and centrifuged at 15 000 × *g* for 40 min. The supernatant was passed through a column of Pro-Bond™ Ni^{2+} affinity resin (Invitrogen). After washing with 20 mM KPB, pH 7.9, containing 0.5 M NaCl and 40 mM imidazole, the recombinant ALS was finally eluted with 15 mM KPB, pH 7.5, containing 10% glycerol and 500 mM imidazole. Finally, to determine subunit composition, the purified enzyme was applied to a HPLC gel filtration column (TSK-gel G3000SW, 7.5 × 600 mm, Tosoh), which was eluted with 100 mM KPB, pH 6.8, containing 10% glycerol and 0.2 M KCl at a flow rate of 1.0 ml/min.

2.5. Site-directed mutagenesis

Cys165 of ALS was replaced by Ser using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and a pair of primers as follows (mutated codons are underlined): C164S sense 5′-ATG GTC TAC CAG CAG GGC TCC TTC GCC GGG G-3′, antisense 5′-CCC CGG CGA AGG AGC CCT GCT GGT AGA CCA T-3′.

2.6. Enzyme reaction

The standard reaction mixture contained 140 nmol of malonyl-CoA (and 54 nmol of other CoA esters) and 220 pmol of the purified recombinant enzyme in a final volume of 500 μl of 100 mM KPB, pH 7.0. Incubations were carried out at 30°C for 90 min, and stopped by adding 50 μl of 20% HCl. The products were then extracted with 1000 μl of ethyl acetate, and analyzed by reverse-phase HPLC and LC-ESI-MS as described before [16]. For large-scale enzyme reaction, 14 mg of purified enzyme was incubated with malonyl-CoA (26 mg, 30 μmol) in 100 ml of 100 mM phosphate buffer, pH 7.0, containing 1 mM EDTA, at 30°C for 20 h. The reaction was quenched by addition of 20% HCl (10 ml), and extracted with ethyl acetate (300 ml × 2). HPLC separation afforded pure aloesone (ca. 0.5 mg). Spectroscopic data: UV: λ_{max} 243, 259, 291 nm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.55 (1H, s, 7-OH), 6.59 (1H, d, J = 2.1 Hz, 6-H), 6.56 (1H, d, J = 2.1 Hz, 8-H), 6.01 (1H, s, 3-H), 3.83 (2H, s, 1′-H), 2.63 (3H, s, 5-Me), 2.19 (3H, s, 3′-Me). EI-MS *m/z* (rel. int.): 232 (M^+, 81), 190 (100), 161 (21), 151 (46), 43 (76).

2.7. Phylogenetic tree

A total of 50 amino acid sequences of CHS superfamily enzymes were aligned and the phylogenetic tree was developed with the CLUSTAL W (1.8) program (DNA Data Bank of Japan, URL: http://www.ddbj.nig.ac.jp) as described before [16,18]. The tree was constructed by use of the majority rule and strict consensus algorithm implanted in PHYLIP [19]. The β-ketoacyl carrier protein synthase III (FABH) of *E. coli* (M96793) was employed as an outgroup [20].

2.8. Three-dimensional homology modeling

The model was produced by the SWISS-MODEL package (http://expasy.ch/spdbv/) provided by the Swiss-PDB-Viewer program [21]. Calculation of cavity volumes (Connolly's surface volumes) was performed with the CASTP program (http://cast.engr.uic.edu/cast/) [22].

3. Results and discussion

A cDNA encoding the novel type III PKS, ALS, was cloned and sequenced from young roots of rhubarb (*R. palmatum*) by reverse transcription PCR amplification using inosine-containing degenerate oligonucleotide primers based on the highly conserved sequences of known CHS enzymes. The terminal sequences of cDNA were obtained by 3′- and 5′-RACE. A 1467-bp full-length cDNA contained a 124-bp 5′ non-coding region, a 1176-bp open reading frame encoding a M_r 43 343 protein with 391 amino acids, and 167 bp of 3′

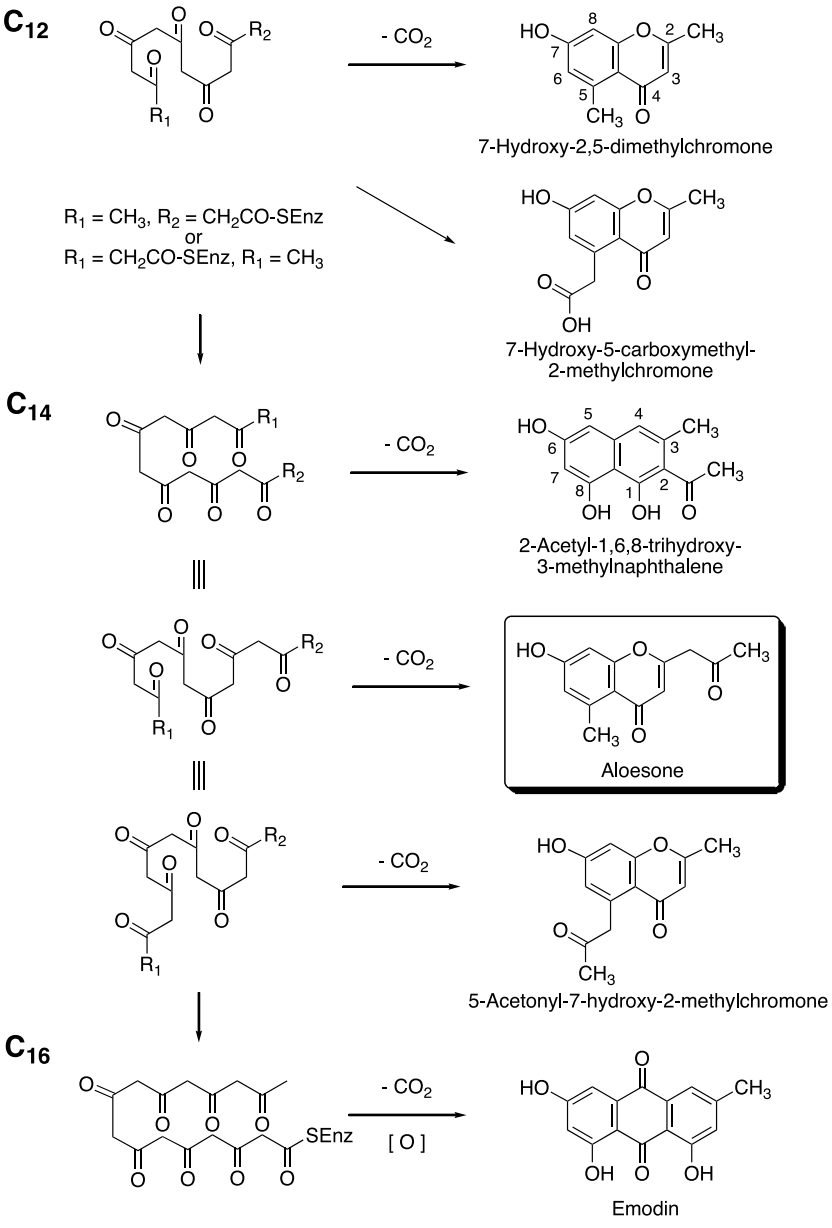

Fig. 2. Proposed biogenesis of aromatic polyketides in rhubarb (modified from [29]). The above listed chromones, naphthalene, and anthraquinone (emodin) have been isolated from rhubarb, suggesting the presence of closely related functionally different PKS enzymes in the plant.

non-coding region (the nucleotide sequence has been deposited in the EMBL/DDBJ/GenBank™ data bases under accession number AY517486).

The deduced amino acid sequence showed 60% identity with those of CHS superfamily enzymes from other plants, 62% identity (243/391) with *M. sativa* CHS (Fig. 3), but only 21% identity (83/391) with RppA from *Streptomyces griseus*, the naphthalene-producing bacterial type III PKS [7]. *R. palmatum* ALS maintains an almost identical CoA binding site and catalytic triad Cys164, His303, and Asn336 (numbering in *M. sativa* CHS) absolutely conserved in all type III PKSs of plant and bacterial origin. Furthermore, most of the active site residues including Met137, Gly211, Gly216, Pro375, along with the CHS 'gatekeeper' phenylalanines, Phe215 and Phe265 [2], are well conserved in ALS. One of the characteristic features is that, in ALS, the CHS active site residues, Thr197, Ile254, Gly256, and Ser338, are uniquely replaced with Ala, Met, Leu, and Thr, respectively. Interestingly, the three residues are also missing in *Gerbera hybrida* 2PS, and a CHS triple mutant (T197L/G256L/S338I) has been reported to yield an enzyme that was functionally identical to 2PS [23]. The CHS superfamily enzymes utilize a common mechanistic strategy that relies on a cysteine residue to anchor the polyketide during a series of condensation reactions. From the sequence alignment, Cys165 in ALS was predicted to correspond to Cys164 in CHS, which was confirmed by site-directed mutagenesis; replacement of Cys165 with Ser resulted in complete loss of enzyme activity.

In the phylogenetic tree (Fig. 4), the heptaketide producing ALS from *R. palmatum* (Polygonaceae) groups with other non-chalcone-forming enzymes including bibenzyl synthase from *Phalaenopsis* sp. (Orchidaceae) [24], two CHS-like enzymes with unknown function from *Ipomoea purpurea* (Convolvulaceae) [25], ACS from *Ruta graveolens* (Rutaceae)

Fig. 3. Comparison of primary sequences of *R. palmatum* ALS and other CHS superfamily enzymes. M.s, *Medicago sativa*; A.h, *Arachis hypogaea*; G.h, *Gerbera hybrida*; R.g, *Ruta graveolens*; R.p, *Rheum palmatum*. The active site residues conserved in the CHS superfamily enzymes (Cys164, Phe215, His303, and Asn336, numbering in *M. sativa* CHS) are marked with #, and residues for CoA binding with +. Amino acid residues conserved in the CHS superfamily enzymes but absent in *R. palmatum* ALS are marked with *.

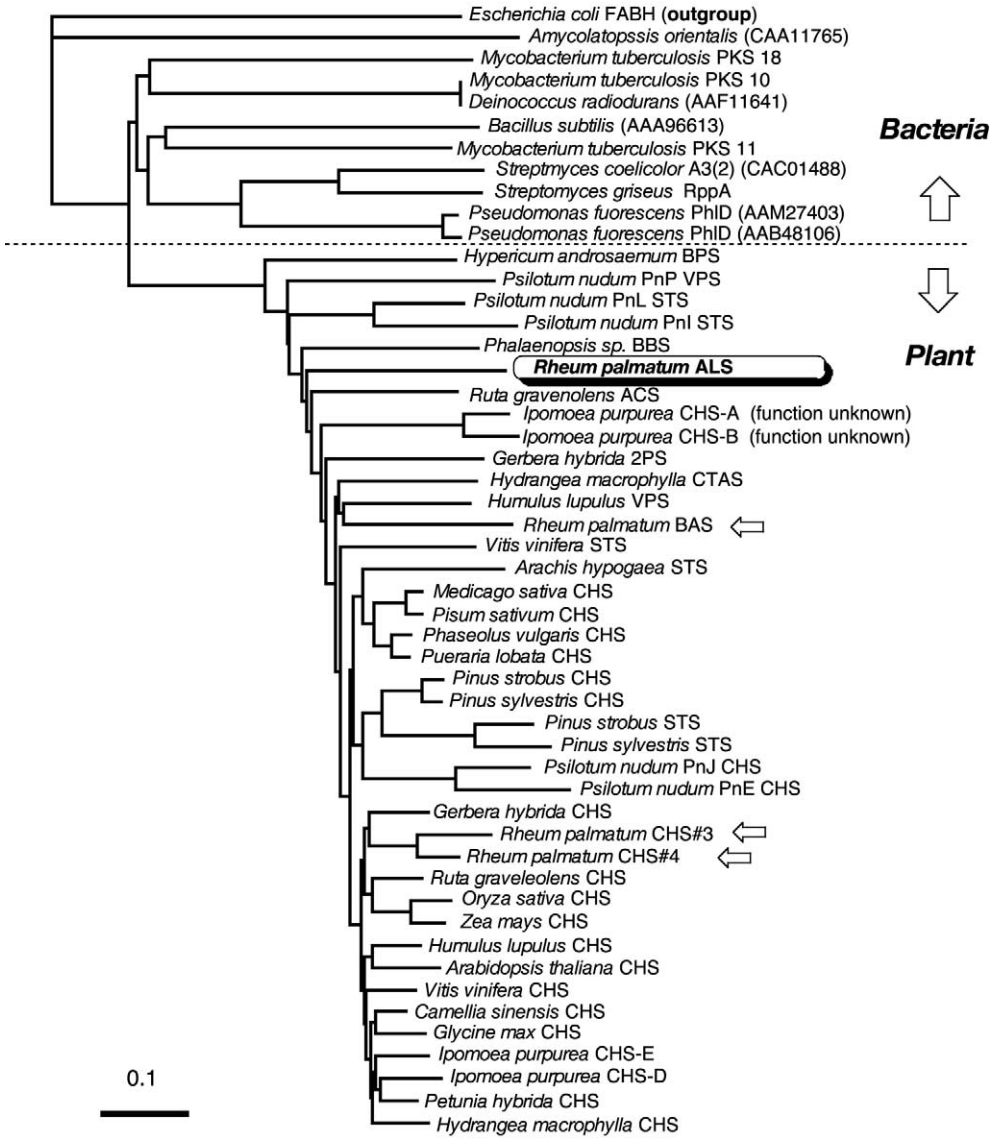

Fig. 4. Phylogenetic analysis of plant and bacterial type III PKS enzymes. Multiple sequence alignment performed by CLUSTAL W (1.8) [18]. The indicated scale represents 0.1 amino acid substitutions per site. Abbreviations: BBS, bibenzyl synthase; BPS, benzophenone synthase; CTAS, 4-coumaroyltriacetic acid synthase; VPS, valerophenone synthase; RppA, red-brown pigment-producing enzyme. Two newly obtained CHS-like clones from *R. palmatum* are also included.

[5], and 2PS from *G. hybrida* (Asteraceae) [6]. On the other hand, two newly obtained CHS-like clones from *R. palmatum* belong to a different cluster. Interestingly, STS, grouping with the CHS from the same or related plants, has been proposed to have evolved independently several times from CHS [20].

The CHS-based homology modeling predicted that *R. palmatum* ALS has the same three-dimensional overall fold as *M. sativa* CHS [8], with a cavity volume (1173 Å^3) slightly larger than that of the chalcone ($C_{15}H_{12}O_5$)-forming CHS (1019 Å^3) but much larger than that of *G. hybrida* 2PS (298 Å^3) [23] that catalyzes formation of a triketide pyrone ($C_6H_6O_3$) from acetyl-CoA and two molecules of malonyl-CoA. This suggests that the active site of ALS is well large enough to perform the six rounds of sequential condensation reactions and to accommodate the heptaketide product ($C_{13}H_{12}O_4$). It has been reported that the shape and volume of the active site cavity greatly influence the substrate specificity of the type III PKS enzymes and control the final length of the polyketide products [23].

Like other type III PKSs, recombinant ALS was functionally expressed in *E. coli* with an additional hexahistidine tag at the C-terminus. The purified enzyme gave a single band with molecular mass of 43 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, while the native ALS appeared to be a homodimer since it had an apparent molecular mass of ca. 90 kDa as determined by HPLC gel filtration. Despite the structural similarity with CHS, the recombinant ALS did not accept either 4-coumaroyl-CoA or other aromatic CoA esters (cinnamoyl-CoA and benzoyl-CoA), but instead efficiently afforded a heptaketide chromone only from seven molecules of malonyl-CoA (unknown minor products were also detected in the reaction mixture). The enzyme showed a $K_M = 86$ μM and $k_{cat} = 27 \times 10^{-3}$ min^{-1}, with a pH optimum within a range of 6.0–8.0. On the other hand, aliphatic CoA esters (butyryl-CoA and hexanoyl-CoA) were also accepted as a substrate,

but with less efficiency. Interestingly, acetyl-CoA, resulting from decarboxylation of malonyl-CoA, was found to be a better starter substrate of ALS, as is the case for *G. hybrida* 2PS [6], and for 6-methylsalicylic acid synthase from *Penicillium patulum* [26], a tetraketide-forming fungal type I PKS that shares no sequence similarity with CHSs. This was confirmed by incubation with [2-^{14}C]malonyl-CoA; the yield of the heptaketide was two- to three-fold higher in the presence of acetyl-CoA. Moreover, it was further supported by the ^{14}C incorporation rate from [1-^{14}C]acetyl CoA in the presence of cold malonyl-CoA. These results suggest that acetyl-CoA is the real starter substrate for ALS. In contrast, *S. griseus* RppA does not accept acetyl-CoA as a starter, but produces the pentaketide naphthalene from five molecules of malonyl-CoA [7].

The structure of the heptaketide product, purified by reverse-phase HPLC, was determined to be aloesone (2-acetonyl-7-hydroxy-5-methylchromone) [27] (Fig. 1B), which was uniquely consistent with both biogenetic reasoning and the following spectroscopic data. First, it showed a MS spectrum with a parent ion peak at *m*/*z* 232 [M$^+$], and a UV spectrum (λ_{max} 243, 259, and 291 nm), suggesting a structure of chromone. Further, the ^{1}H NMR of the enzyme reaction product obtained from large-scale incubation (ca. 0.5 mg from 26 mg of malonyl-CoA) revealed one olefinic proton (δ 6.01), two *meta*-coupled aromatic protons (δ 6.59 and 6.56), and one aromatic methyl singlet (δ 2.63). In addition, there were an acetyl (δ 2.19) and an isolated methylene (δ 3.83), attributable to an acetonyl group. Confirmation of the structure was finally obtained by direct comparison (GC, GC-MS, and ^{1}H NMR) with an authentic aloesone isolated from commercial aloin powder.

R. palmatum ALS is the first plant-specific type III PKS that catalyzes the formation of an aromatic heptaketide from acetyl-CoA and six molecules of malonyl-CoA. The six successive decarboxylative polyketide chain elongations and subsequent regiospecific cyclization of the heptaketide intermediate with the removal of a carboxyl group from the carboxyl end lead to the formation of aloesone (2-acetonyl-7-hydroxy-5-methylchromone) (Fig. 1B). It is quite remarkable that assembly of such a complex aromatic heptaketide is catalyzed by the relatively simple homodimeric type III PKS, structurally and mechanistically quite distinct from the type I (modular type) and type II (subunit type) PKSs. In contrast, in bacteria, such long-carbon-chain ($>C_{10}$) polyketides are synthesized only by the type I or type II PKS enzymes with the involvement of 4′-phosphopantetheine residues on acyl carrier protein.

Finally, it should be noted that isolation of aloesone *O*-glucoside (7-*O*-β-D-glucopyranoside) has been reported from rhubarb (Polygonaceae) [28]. Further, it is also known that aloe (*Aloe ferox*, Liliaceae) contains significant amounts of aloesone *C*-glucoside (8-*C*-β-D-glucopyranoside) (aloesin), for which anti-inflammatory effects have been reported [27]. In addition to aloesone, these medicinal plants produce a variety of aromatic polyketides (e.g. chromones, naphthalenes, phenylbutanones, stilbenes, and anthraquinones) (Fig. 2), suggesting the presence of closely related functionally different enzymes [29]. It is thus likely that the CHS superfamily of type III PKSs may be rather widely involved in the biogenesis of plant secondary metabolites, possibly including the octaketide-derived anthraquinones. In order to understand the structure–function relationship between ALS and other CHS superfamily enzymes, further characterization of the enzyme is now in progress.

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