



## Characterization of olivetol synthase, a polyketide synthase putatively involved in cannabinoid biosynthetic pathway<sup>☆</sup>

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### ABSTRACT

Alkylresorcinol moieties of cannabinoids are derived from olivetolic acid (OLA), a polyketide metabolite. However, the polyketide synthase (PKS) responsible for OLA biosynthesis has not been identified. In the present study, a cDNA encoding a novel PKS, olivetol synthase (OLS), was cloned from *Cannabis sativa*. Recombinant OLS did not produce OLA, but synthesized olivetol, the decarboxylated form of OLA, as the major reaction product. Interestingly, it was also confirmed that the crude enzyme extracts from flowers and rapidly expanding leaves, the cannabinoid-producing tissues of *C. sativa*, also exhibited olivetol-producing activity, suggesting that the native OLS is functionally expressed in these tissues. The possibility that OLS could be involved in OLA biosynthesis was discussed based on its catalytic properties and expression profile.

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### 1. Introduction

Cannabinoids, which are found only in *Cannabis sativa*, are unique secondary metabolites consisting of alkylresorcinol and monoterpene groups. More than 60 cannabinoids have been isolated from marijuana or fresh *Cannabis* leaves [1], and their pharmacological properties and biosynthetic mechanisms have been investigated extensively [2,3]. With respect to the biosynthesis of cannabinoids, it has been demonstrated that the major cannabinoids (tetrahydrocannabinolic acid and cannabidiolic acid) are biosynthesized by novel FAD-dependent oxidases named tetrahydrocannabinolic acid synthase and cannabidiolic acid synthase, respectively, from the common precursor cannabigerolic acid [4,5]. In addition, Fellermeier and Zenk identified a geranyltransferase activity producing cannabigerolic acid from OLA and

geranylpyrophosphate, suggesting that the alkylresorcinol moieties of cannabinoids are derived from OLA [6].

OLA is a kind of resorcinolic acid and has been assumed to be biosynthesized via the polyketide pathway [7]. In the proposed biosynthetic mechanism (Fig. 1A), OLA is synthesized by a polyketide synthase (PKS)-type reaction from hexanoyl-CoA and three molecules of malonyl-CoA via an aldol condensation of a tetraketide intermediate. However, because the PKS catalyzing OLA biosynthesis has been neither identified nor cloned, the biosynthetic mechanism of OLA remains unclear.

In contrast, recent structure-function analyses of plant PKSs have suggested that numerous biosynthetic enzymes are evolved from chalcone synthase (CHS, Fig. 1B), the ubiquitous plant type III PKS catalyzing the first committed step in flavonoid biosynthesis, by changing active site residues regulating substrate specificity and/or cyclization reactions of linear polyketide intermediates [8]. For example, crystal structure analyses of CHS and stilbene synthase (STS, Fig. 1B) have suggested that only a small number of amino acid substitutions in CHS alter the cyclization reaction from claisen-type into aldol-type, and that STS evolved from CHS with this functional change called the aldol switch [9,10]. Thus, it was assumed that the PKS responsible for OLA biosynthesis may also be derived from CHS to open a metabolic entrance into the cannabinoid pathway.

**Abbreviations:** CHS, chalcone synthase; NAC, *N*-acetylcysteamine; OLA, olivetolic acid; OLS, olivetol synthase; PKS, polyketide synthase; RACE, rapid amplification of cDNA ends; STS, stilbene synthase

<sup>☆</sup> Nucleotide sequence data of OLS is available in the DDBJ/EMBL/GenBank databases under the accession number AB164375.

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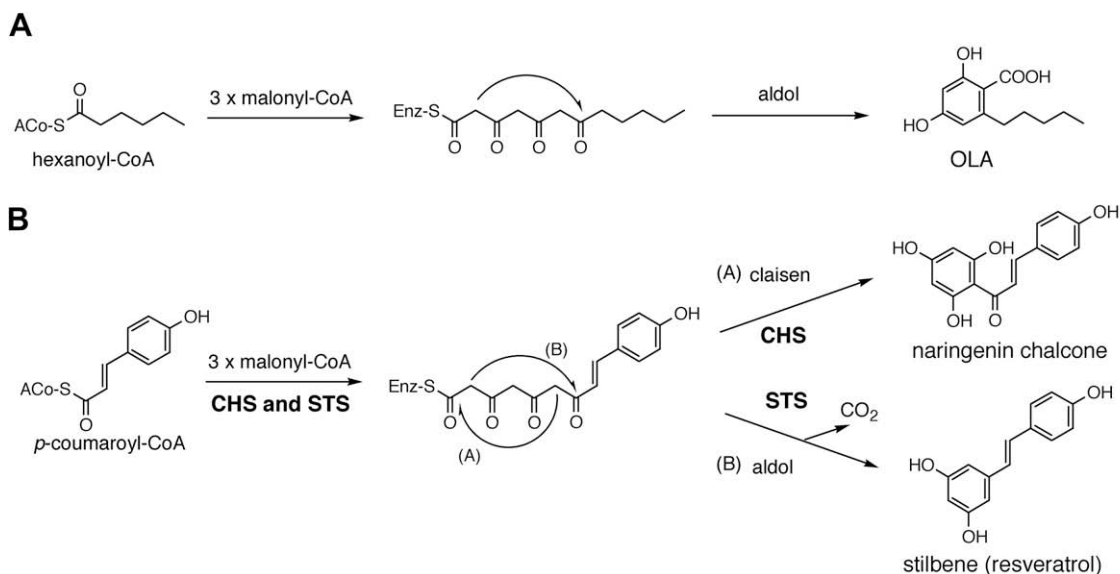


Fig. 1. The proposed biosynthetic reaction of OLA (A) and the reactions catalyzed by CHS and STS (B).

In the present study, a gene encoding a novel type III PKS was cloned from *C. sativa* and named olivetol synthase (OLS). The catalytic properties of OLS were characterized with *N*-acetylcysteamine (NAC) substrate analogs as well as CoA substrates. In addition, the expression profile of OLS in various parts of *Cannabis* plants was also herein investigated.

## 2. Materials and methods

### 2.1. Plant materials and chemicals

*C. sativa* plants (Mexican strain) were cultivated in the herbal garden of the Graduate School of Pharmaceutical Sciences, Kyushu University. Olivetolic acid (OLA) was prepared from olivetol (Sigma) with methylmagnesium carbonate as described previously [4]. *p*-Coumaroyl-CoA was synthesized according to the reported method [11]. NAC thioesters of fatty acids were synthesized according to the method [12]. Malonyl-NAC was prepared following the procedure to synthesize malonyl-*N*-caprylcysteamine [13], using NAC instead of *N*-caprylcysteamine. All other chemicals were purchased from Sigma and Wako (Osaka, Japan), and molecular biology reagents were from Takara (Tokyo, Japan) and Toyobo (Osaka, Japan), unless otherwise stated.

### 2.2. RNA extraction and reverse transcription

Total RNA was extracted from rapidly expanding leaves of *C. sativa* as described previously [4]. The first strand cDNA was synthesized using reverse transcriptase and an oligo(dT) primer (primer j below). cDNA with poly(dA) tail at 3'-terminus was prepared with terminal deoxynucleotidyl transferase in the presence of deoxyadenine.

### 2.3. Cloning and sequencing of cDNA encoding OLS

The following oligonucleotide primers were used in this study: degenerate primers a (5'-GGGGTACCATHHTYTGGATHGCICAYC-3'), b (5'-GCTCTAGAARICCNCGICRAAICCRA-3'), and c (5'-CGGGATCC-RMITAYCCIGAYTAYTAYT-3'), designed from peptide sequences, IFWIAHP, FGFPGPL, and YPDYF, respectively; gene-specific primers d (5'-GGGGTACCAGCACATGACGTGAATCCA-3'), e (5'-GGGGTA-

CCTGAGTTGAGGAAGAGGTCG-3'), f (5'-CAGTTACGTTTCCTTATCA-TAC-3'), g (5'-GGGGTACCCGAACTTTCTTTGAGTTGAG-3'), h (5'-CGGGATCCCATATGAATCATCTTC-GTGCTG-3'), and i (5'-TATCTTGT-CGACATATTTGATGGGAACACT-3'); and adapter primers j (5'-GAC-TCGTCTAGAGGATCCCG(T)<sub>17</sub>-3') and k (5'-GACTCGTCTAGAGGAT-CCCG-3').

All cDNA fragments were amplified by PCR using *Taq* DNA polymerase. First, the core fragment (~240 bp) was obtained by PCR with degenerate primers a and b. The cDNA fragment for the 5'-upstream region up to near the protein N-terminus (~900 bp) was amplified with primers c and d. The 3'-terminal and 5'-terminal regions were amplified by rapid amplification of cDNA ends (RACE) [14]. The 3'-RACE product (~370 bp) was obtained by PCR with gene-specific primer e and adapter primer k. 5'-RACE product (~200 bp) was amplified as follows. The first round of PCR was performed with gene-specific primer f and adapter primer j in the presence of poly(dA)-tailed cDNA. The cDNA fragment was obtained by the nested PCR with gene-specific primer g and adapter primer k. All PCR products were cloned into the vector pUC119 and sequenced using an Applied Biosystems 310 genetic analyzer.

### 2.4. Expression and purification of the recombinant OLS

Full-length cDNA was amplified using gene-specific primers h and i with a proofreading polymerase (KOD DNA polymerase, Toyobo). The amplified cDNA was digested with *Bam*HI and *Sal*I, and subcloned into pUC119 vector for DNA sequencing. Then, the cDNA fragment with the correct sequence was excised with *Nde*I and *Sal*I, and ligated into a pET24a expression vector predigested with *Nde*I and *Xho*I. The resulting construct, which directed the synthesis of the recombinant OLS with a C-terminal hexahistidine tag, was transformed into *Escherichia coli* BL21 (DE3).

Cells harboring the recombinant plasmid were cultured in liquid LB medium (1000 ml) containing 25 µg/ml kanamycin. When the optical density of the culture at 660 nm reached 0.6, isopropyl-β-D-thiogalactoside (0.4 mM) was added to the culture to induce the recombinant protein expression. After incubation at 25 °C for 5 h, the cells were harvested by centrifugation, resuspended in 50 ml of buffer A (20 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl), and disrupted by sonication. The homogenate was centrifuged at 20 000×g for 20 min to remove insoluble materials.

The supernatant was applied to a column (1.0 × 10 cm) containing 5 ml of His-bind resin (Novagen) equilibrated with buffer A. After sample application, non-specifically bound proteins were removed with 10 column volumes of buffer A containing 120 mM imidazole. Then, hexahistidine-tagged OLS was eluted with three column volumes of buffer A containing 250 mM imidazole. The purity of OLS was verified by SDS-PAGE analysis. The native molecular mass of OLS was determined by gel filtration chromatography on a 2.5 × 75-cm column of Sephacryl S-200 HR (GE Healthcare) calibrated with standard proteins.

### 2.5. Standard assay conditions of OLS

The standard reaction mixture consisted of 100 μM hexanoyl-CoA, 200 μM malonyl-CoA, and 100 mM sodium citrate buffer (pH 5.5) in a total volume of 160 μl. The reaction was started by adding 40 μl of enzyme solution, and the mixture was incubated at 30 °C for 30 min. After termination of the reaction with 200 μl of methanol, a 100-μl aliquot was subjected to analysis using the HPLC system, as reported previously [4], equipped with a column Cosmosil 5C18-AR (4.6 × 150 mm, Nacalai Tesque, Tokyo, Japan). The reaction products were eluted with 40% aqueous acetonitrile containing 50 mM phosphoric acid at a flow rate of 1 ml/min, and detected by absorption at 280 nm. When starter substrates other than hexanoyl-CoA were used, the concentration of acetonitrile was changed to detect each reaction product as appropriate.

### 2.6. Enzyme kinetics

Enzyme assays were carried out with 200 μM malonyl-CoA and various concentrations of starter substrates. The kinetic parameters were determined by Lineweaver-Burk double-reciprocal plots of the velocity curve of the polyketide-formation reactions.

### 2.7. Large-scale reaction from NAC-thioesters and structural analyses of products

In the presence of purified enzyme (5 mg), the substrate buffer (1000 ml), which was composed of 100 mM sodium citrate buffer (pH 5.5), 1 mM hexanoyl-NAC, and 2 mM malonyl-NAC, was incubated for 3 h at 30 °C. The reaction mixture was partitioned with AcOEt–AcOH (2:1), and the organic layer was evaporated. Preparative HPLC, in which a Cosmosil 5C-18AR (10 × 200 mm) column was used, gave three reaction products; olivetol (7.1 mg), tetraketide pyrone (1.9 mg), and triketide pyrone (5.4 mg). Olivetol (5-pentylresorcinol): FAB-MS:  $m/z$  181 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 6.22 (2H, *d*, *J* = 2.0 Hz, H-4, 6), 6.17 (1H, *t*-like, *J* = 2.0 Hz, H-2), 2.45 (2H, *t*, *J* = 6.0 Hz, H-1'), 1.57 (2H, *m*, H-2'), 1.32 (4H in total, *m*, H-3', 4'), 0.87 (3H, *t*, *J* = 6.0 Hz, H-5'). Tetraketide pyrone (4-hydroxy-6-(2'-oxo-heptyl)-2-pyrone): FAB-MS:  $m/z$  225 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 5.98 (1H, *d*, *J* = 2.0 Hz, H-5), 5.47 (1H, *d*, *J* = 2.0 Hz, H-3), 3.53 (2H, *s*, H-1'), 2.52 (2H, *t*, *J* = 6.0 Hz, H-3'), 1.60 (2H, *m*, H-4'), 1.26 (4H in total, *m*, H-5', 6'), 0.87 (3H, *t*, *J* = 6.0 Hz, H-7'). Triketide pyrone (4-hydroxy-6-pentyl-2-pyrone): FAB-MS:  $m/z$  183 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 5.92 (1H, *d*, *J* = 2.0 Hz, H-5), 5.52 (1H, *d*, *J* = 2.0 Hz, H-3), 2.44 (2H, *t*, *J* = 6.0 Hz, H-1'), 1.62 (2H, *m*, H-2'), 1.33 (4H in total, *m*, H-3', 4'), 0.87 (3H, *t*, *J* = 6.0 Hz, H-5').

In a similar manner, reaction products were isolated from the respective reactions of butyryl-NAC, isovaleryl-NAC, and octanoyl-NAC as the starter substrates.

### 2.8. Protein extraction from *C. sativa* plants

Twenty-week-old female plants were used for protein extraction. Plant materials from various tissues (5 g) were ground to fine

powders in the presence of liquid nitrogen using a pestle and mortar. The proteins were extracted with 20 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 10 mM mercaptoethanol, then centrifuged to remove insoluble materials. The supernatants were applied to PD-10 columns (GE Healthcare) prebuffered with 10 mM sodium phosphate buffer (pH 7.0) containing 3 mM mercaptoethanol. The enzyme activity was assayed as described above.

### 2.9. Preparation of polyclonal anti-OLS antibody and Western blotting

Polyclonal antiserum against OLS was generated in white female rabbits using 1 mg of purified OLS per injection. The antiserum obtained after the third booster was applied to an affinity column containing CNBr-activated Sepharose 4B-OLS conjugate, and anti-OLS antibody was purified as described by the manufacturer (GE Healthcare). For Western blotting analysis, *C. sativa* protein extracts were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was incubated at room temperature for 1 h with affinity-purified anti-OLS antibody, and horseradish peroxidase-conjugated secondary antibodies. The protein bands were visualized using Immunostaining HRP-1000 reagent (Seikagaku Corp., Tokyo, Japan).

## 3. Results and discussion

### 3.1. Cloning and sequencing of a cDNA encoding OLS

The cDNA encoding OLS was cloned by reverse transcription-PCR from expanding leaves of *C. sativa*. First, a 240-bp cDNA fragment was obtained by PCR using degenerate primers designed from conserved sequences in CHS and related plant PKs. After sequence determination of the 240-bp DNA, the amplification of cDNA fragments containing the 3'-downstream and 5'-upstream regions was conducted by 3'- and 5'-RACE, respectively. Then, a PCR reaction with gene-specific primers successfully amplified the full-length cDNA.

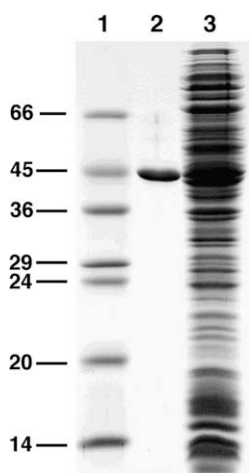
The gene consisted of a 1185-bp open reading frame encoding a 385-amino acid polypeptide with a molecular mass of 42 585 Da. The deduced amino acid sequence showed high (60–70%) identity to those of plant PKs. In addition, the identity between OLS and *Medicago sativa* CHS, of which the crystal structure and reaction mechanism has been characterized extensively [9], was also significant (~65%) (Fig. 2). Furthermore, the catalytic triad residues of CHS, Cys164-His303-Asn336, which coordinately catalyze the chain elongation reactions, were conserved in the corresponding positions of OLS (Fig. 2). These structural features suggested that OLS is a CHS-related enzyme that catalyzes polyketide formation with a mechanism similar to CHS. However, it was of interest that OLS contained some amino acid differences in the CHS active site [9]. For example, Thr132, Thr194, and Thr197 in CHS, the proposed important residues for catalytic diversity [15], were substituted into Ala, Met, and Leu in OLS, respectively (Fig. 2). Thus, it was regarded that OLS could be a novel enzyme derived from CHS.

### 3.2. Bacterial expression and characterization of the recombinant OLS

Recombinant OLS was expressed in *E. coli* with a C-terminal hexahistidine tag, and purified by metal chelate affinity chromatography. SDS-PAGE analysis demonstrated that the recombinant enzyme was purified as a homogeneous ~44 kDa protein (Fig. 3). The native molecular mass of OLS was estimated to be ~89 kDa by a gel filtration chromatography, indicating that OLS is a homodimeric protein. Using the purified enzyme, the enzyme activity was assayed with hexanoyl-CoA and malonyl-CoA, the expected substrates for the OLA biosynthetic reaction. Consequently, OLS

OLS	1	-----MNHLRAEGPASVLAIGTANPENILLQDEFDPDYFRVTKSEHMTOLKEKFRKIC	53
CHS	1	MVSVSEIRKAQRAEGPATILLAIGTANPANCVVEQSTYPDFYFKITINSEHKTTELKEKFRQRC	60
OLS	54	DKSMIRKRNCFLNEEHLKONPRLVEHEMOTLDARQDMLVVEVPKLGKDACAKAIKEWGQP	113
CHS	61	DKSMIKRRMYLTEEILKENPNVCEYMAPSLDARQDMVVVEVPRLGKEAAVKAIKEWGQP	120
OLS	114	KSKITHLIFTASSTTDMPGADYHCAKLLGLSPSVKRVMMYQLGCYGGTVLRIAKDIAEN	173
CHS	121	KSKITHLIVCTTSQVDMPGADYQLTKLLGLRPYVKRYMMYQGGFAGGTVLRLAKDLAEN	180
OLS	174	NKGARVLAVCCDIMACLFRGPSESDLELLVGQALIFGDGAAAVIVGAEPDESVMGERPIFEL	233
CHS	181	NKGARVLVVCSEVTAVTFRGPSDTHLDSLVGQALIFGDGAAALIVGSDPVPEI-EKPIFEM	239
OLS	234	VSTGQTILPNSEGTGGHIREAGLIFDLHKDVPMLISNNIEKCLIEAFTPIGIGSDWNSIF	293
CHS	240	VWTAQTIIAPDSEGATDGHIREAGLTFHLLKDVPGIVSKNITKALVEAFEPGLGSDYNSIF	299
OLS	294	WITHPGGKATLDKVEEKLHLKSDKIFVDSRHVLSHGMMSSSTVLFVMDLRRKRSLEEGLKS	353
CHS	300	WIAHPGGPATLDQVEQKLALKPEKMNATREVLSEYGNMSSACVLFITLDEMRRKSTQNLK	359
OLS	354	TTGDGFIEWGVLFVGFPGPLTVERVVRVPIKY	385
CHS	360	TTGEGLEWGVLFVGFPGPLTIETVLRVVAI--	389

**Fig. 2.** Comparison of the primary sequences of *C. sativa* OLS and *Medicago sativa* CHS. The catalytic triad residues in CHS (Cys164, His303, and Asn336, numbering in *M. sativa* CHS) are marked with #. The CHS active site residues that are proposed to be important for substrate specificity or polyketide size control [15] were indicated with \*.



**Fig. 3.** SDS-PAGE analysis of the purified recombinant OLS. Lane 1, molecular mass standards; lane 2, 3  $\mu$ g of the purified OLS; and lane 3, total soluble proteins from *E. coli* BL21(DE3) expressing OLS.

yielded three reaction products. However, unexpectedly, none of these were OLA judging from their retention time on HPLC analysis.

To obtain an adequate amount of polyketide products for structural analyses, sufficient amounts of substrate analogs were synthesized chemically from NAC, a cost-effective small molecule with a similar structure to the reactive portion of CoA [16]. Then, a large-scale incubation using NAC thioesters with OLS was attempted. As a result, OLS catalyzed the reaction from hexanoyl-NAC and malonyl-NAC, and afforded the same product profile as the reaction from hexanoyl-CoA and malonyl-CoA. Recently, several groups have reported that acyl-NACs could prime the reactions of type III PKSs [17,18]; however, these studies did not exploit the extender substrate analogs such as malonyl-NAC. Thus, this was the first enzymatic synthesis of polyketides by a type III PKS only from synthetic substrate analogs.

The resulting three products were isolated by preparative-HPLC with milligram quantities, and their structures were analyzed by  $^1\text{H}$  NMR and FAB-MS analyses. As illustrated in Fig. 4, it revealed that OLS synthesizes two tetraketide-derived products, olivetol and tetraketide pyrone, together with triketide pyrone. Tetraketide and triketide pyrones were reported to be the reaction products of

various type III PKSs [19–22], and triketide pyrone could be a derailment product from a premature intermediate. Meanwhile, to our knowledge, OLS is the first plant PKS producing tetraketide alkylresorcinol such as olivetol to be cloned. Olivetol is the decarboxylated form of OLA; however, it is not likely that OLS synthesizes olivetol via OLA because the present study could not detect OLA although various assay and HPLC conditions were tested. In addition, incubation of OLA with OLS under standard assay conditions did not produce olivetol.

Next, using enzymatically-synthesized polyketides as standard compounds, characterization of the enzyme reactions with CoA substrates was attempted. As shown in Table 1, kinetic analysis of OLS reactions from hexanoyl-CoA demonstrated that this enzyme synthesized olivetol with a higher  $k_{\text{cat}}/K_m$  value ( $1013 \text{ s}^{-1} \text{ M}^{-1}$ ) than those for tetraketide pyrone and triketide pyrone syntheses ( $280$  and  $811 \text{ s}^{-1} \text{ M}^{-1}$ , respectively). These data suggest that OLS catalyzes decarboxylative-aldol condensation with higher efficiency than that for lactonization.

STS, which produces resveratrol from *p*-coumaroyl-CoA and malonyl-CoA, also catalyzes the same cyclization reaction as olivetol formation. Recently, crystal structural analysis of STS demonstrated that STS contains a thioesterase-like domain to catalyze hydrolytic release of the linear tetraketide intermediate anchored at the active site cysteine, and this liberation of tetraketide into the enzyme active site leads to the decarboxylative-aldol condensation to form resveratrol [10]. Thus, the active site of OLS should also have structural features to catalyze thioester hydrolysis as well as aldol condensation of the tetraketide intermediate primed by hexanoyl-CoA. On the other hand, it has also been reported that STS synthesizes only pyrone products when incubated with aliphatic CoA starters including hexanoyl-CoA [20]. Therefore, the active-site architecture of OLS could be somewhat different from that of STS.

With respect to substrate specificity, besides hexanoyl-CoA, OLS accepted starter CoA esters with C4 to C8 side chains such as butyryl-, isovaleryl-, and octanoyl-CoA; however, it produced triketide pyrones from these substrates except affording 5-propylresorcinol (divarinol) from butyryl-CoA with a lower  $k_{\text{cat}}/K_m$  value ( $129 \text{ s}^{-1} \text{ M}^{-1}$ ) than that for olivetol formation (Table 1). In addition, this enzyme did not catalyze any reactions from aromatic CoA esters including *p*-coumaroyl-CoA, a typical starter substrate for plant PKSs. These results indicated that OLS exclusively accepts starter CoA esters with C4 to C8 aliphatic side chains and mostly preferred hexanoyl-CoA. The substrate specificity of OLS was

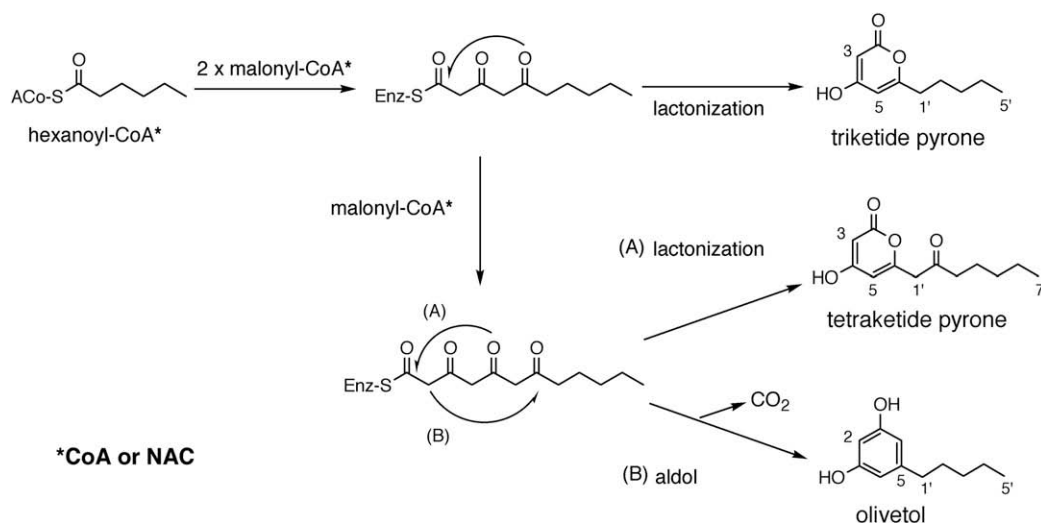


Fig. 4. The reaction catalyzed by OLS.

**Table 1**  
Kinetic parameters of OLS for various starter substrates.

Substrate	Triketide pyrone			Tetraketide pyrone			Resorcinol		
	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{s}^{-1} \text{M}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{s}^{-1} \text{M}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{s}^{-1} \text{M}^{-1}$ )
Hexanoyl-CoA	60.8	2.96	811	63.1	1.06	280	56.9	3.46	1013
Butyryl-CoA	88.9	0.719	134	–	–	–	86.1	0.667	129
Isovaleryl-CoA	99.1	0.585	98.4	–	–	–	–	–	–
Octanoyl-CoA	81.7	0.525	107	–	–	–	–	–	–

Data are means of triplicate assays, and the standard deviation was always within 10%.

‘–’ Activity not detected.

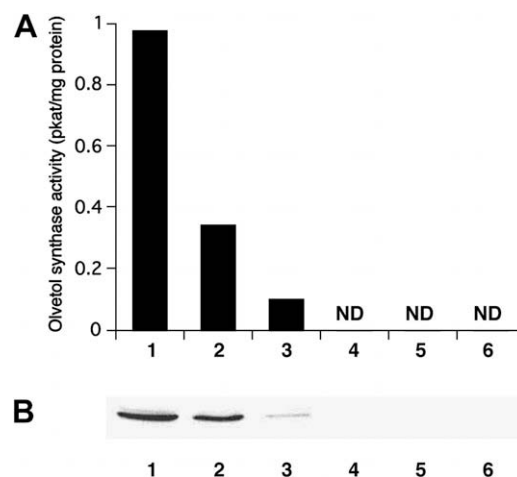
relatively restricted compared with most plant PKSs, including CHS and STS, which exhibit highly promiscuous substrate specificity to react with both of various aliphatic and aromatic CoA starter molecules [19,20]. Therefore, the substrate-binding site of OLS could be suitably shaped by amino acid substitutions as described above to select hexanoyl-CoA. Crystal structural analysis and site directed mutational studies of OLS are now in progress to probe the structural basis for the unique substrate specificity and catalytic properties of this enzyme.

### 3.3. Expression of the native OLS in *C. sativa* plants

As described, OLA formation was not detected in OLS assays. However, it was also confirmed that the crude enzyme extracts prepared from flowers and rapidly expanding leaves, the cannabinoid-producing tissues of *C. sativa* [7], also exhibited activities similar to OLS; the crude enzymes did not synthesize OLA, but yielded olivetol as shown in Fig 5A. In addition, Western blotting analysis using an anti-OLS antibody clearly indicated the expression of OLS in flowers and rapidly expanding leaves (Fig. 5B). These results suggested that the native OLS was expressed in these cannabinoid-producing tissues as a catalytically active enzyme whereas OLA-producing activity was not detected in any samples. It was considered that these data raised a possibility that OLA biosynthesis may not be catalyzed only by a PKS. In addition, olivetol may be an artifact of in vitro enzyme assays because olivetol is not detected in *Cannabis* tissues (F. Taura, unpublished observation).

Notably, Funa et al. has recently proposed an interesting mechanism for resorcinolic acid biosynthesis by a fungal type III PKS (2'-oxoalkylresorcylic acid synthase); they hypothesized that resorcinolic acid is produced when aldol condensation of the intermediate

takes place prior to hydrolytic release from the active site cysteine contrary to the STS-type reaction, which proceeds through a hydrolysis-cyclization scheme [23]. Thus, in *Cannabis* plants, there might be unknown factors that affect the timing of the hydrolysis and cyclization reactions by OLS, to form an OLA-forming metabolic complex together with OLS. Metabolon formation has actu-



**Fig. 5.** Analyses of the expression of OLS in *C. sativa*. (A) Olivetol-producing activity in crude extracts from various tissues. Numbers indicate flowers (1), rapidly expanding leaves (2), young leaves (3), mature leaves (4), stems (5), and roots (6). The data are means of triplicate assays, and the standard deviation was always within 10%. pkat, picokatal. ND, Activity not detected. (B) Western blotting analysis of crude extracts from various tissues using anti-OLS polyclonal antibody. Numbers indicate the same samples as shown in panel A.

ally been reported for a plant type III PKS. It has been demonstrated that *Arabidopsis thaliana* CHS interacts with chalcone isomerase and dihydroflavonol 4-reductase for efficient and stereoselective flavonoid biosynthesis via metabolic channeling [24].

In summary, the present study characterized OLS, a novel plant type III PKS from *C. sativa*. OLS could be an appropriate candidate for the PKS involved in OLA biosynthesis, because OLS is specific to hexanoyl-CoA, catalyzes aldol condensation, and is actually expressed in *Cannabis* plants. Further studies should be performed to clarify the biosynthetic mechanism of OLA and physiological function of OLS in *C. sativa*. In addition, it was also of interest that OLS could synthesize polyketides from cost-effective substrate analogs. Similar enzymatic synthesis using various PKSs may lead to the development of a polyketide product library for surveying novel medicinal resources, because plant polyketides, such as chalcones and stilbenes, show valuable pharmacological activities [25,26].

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