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Incorporation of three deuterium atoms excludes intermediacy of stilbenecarboxylic acid in stilbene synthase reaction

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Abstract—To investigate the mechanism of stilbene synthase (STS) reaction, the origin of the aromatic protons of resveratrol B-ring was examined using STS from *Arachis hypogaea* expressed in *Escherichia coli* and deuterated malonyl-CoA. The presence of resveratrol labeled with three deuterium atoms was detected by GCMS analysis indicating decarboxylation earlier than aromatization, i.e. exclusion of intermediacy of stilbenecarboxylic acid in STS reaction.

The formation of naringenin chalcone, the first pathway-specific reaction of flavonoid biosynthesis catalyzed by chalcone synthase (CHS), involves the assembly of one molecule of *p*-coumaroyl–CoA and three molecules of malonyl–CoA into a tetraketide intermediate, its cyclization (connecting carbon 1 and 6) and enolization to produce the final structure (Fig. 1). Resveratrol, which has a skeleton different from that of chalcone, is formed by stilbene synthase (STS) with the same substrates, but in a different manner (connecting carbon 2 and 7 and concomitant decarboxylation). Both enzymes share common features in structure and function and form a family, called CHS superfamily, together with other CHS-related enzymes.[1] It is interesting that these different modes of cyclization produce structural diversity of secondary metabolites.

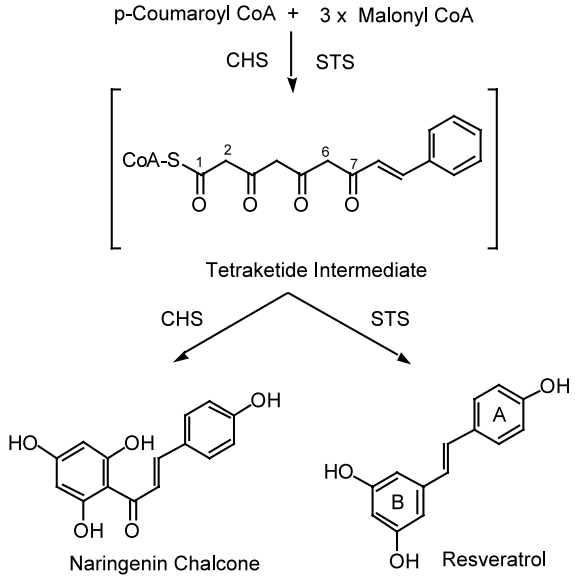

Figure 1. Reaction of chalcone and stilbene synthases.

To investigate the reaction mechanism of plant polyketide synthases, a survey of by-products,[2,3] analysis of the decarboxylation mechanism of malonyl–CoA,[4,5] and examination of substrate specificity[6] have been extensively carried out. However, no substantial information has been obtained on the cyclization mechanism of the tetraketide intermediate into the stilbene skeleton. The mechanism via stilbenecarboxylic acid (mechanism A in Fig. 2) was proposed by Schröder,[7] based on the presence of this type of compounds in the plant kingdom.

If resveratrol is assumed to be formed via stilbenecarboxylic acid, stilbenecarboxylic acid could be detected only in the STS reaction mixture and not in the CHS reaction. As reported in our previous paper,[3] in vitro reaction products of recombinant CHS from *Pueraria lobata* and STS from *Arachis hypogaea*, both expressed in *Escherichia coli*, were extensively analyzed. In addition to the intrinsic products and cross-reaction products (resveratrol for CHS and naringenin chalcone for STS), purified CHS and STS produced bisnoryangonin and *p*-coumaroyltriacetic acid lactone (CTAL) as

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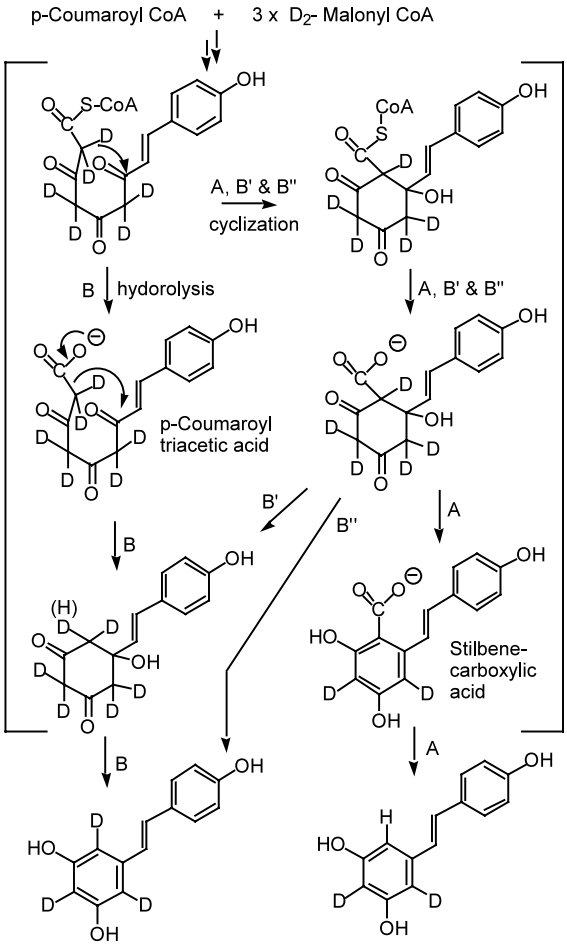

Figure 2. Possible mechanisms of stilbene synthase reaction.

common by-products. In these experiments, however, no STS-specific by-products including stilbenecarboxylic acid were detected even with total ion monitoring by LC–MS. Polyketide reductase from *P. lobata*, which co-acts with CHS to produce deoxy-type chalcone, was added to the STS reaction mixture with NADPH in the expectation that hydrangeic acid, a deoxy-type stilbenecarboxylic acid, would be produced. However, neither deoxy-type stilbene nor stilbenecarboxylic acid was detected (unpublished data).

These observations led us to assume three alternative mechanisms for resveratrol formation, which do not involve stilbenecarboxylic acid as an intermediate, i.e. a simultaneous decarboxylation and cyclization of the free tetraketide carboxylate intermediate (Fig. 2, mechanism B), decarboxylation before aromatization (mechanism B′), and simultaneous decarboxylation and aromatization (mechanism B″). It is very difficult to distinguish one from the others. But these mechanisms could be clearly distinguished from mechanism A by an incorporation experiment using [D_2]–malonyl–CoA. If mechanism A is operating, only two positions of resveratrol would be labeled with deuterium, while in mechanism Bs (B, B′ and B″), three positions could be labeled. Consequently, two mass unit shifts of molecular ion could be observed with mechanism A, and a three-unit shift in mechanism Bs, when the labeled resveratrol is analyzed by mass spectrometry.

[D_2]–Malonyl–CoA was prepared from malonyl–CoA and D_2O (99.9 atom% D, ISOTEC) by proton–deuterium exchange.[8] Ten milligrams of malonyl–CoA (Sigma) was dissolved in 4 ml of D_2O, maintained at room temperature for 3 h and lyophilized. This procedure was repeated and the resulting material was stored in a desiccator until use. The reaction mixture consisting of *p*-coumaroyl–CoA (200 nmol) and purified STS (537 pkat)[3] in 0.9 ml of 0.1 M Tris HCl buffer (pH 8.0) containing 1 mM EDTA was preincubated at 30°C for 2 minutes. Reaction was started by adding 0.1 ml of 10 mM [D_2]–malonyl–CoA, which was prepared just prior to the experiment by dissolving in D_2O. After 2 min incubation at 30°C, the reaction was stopped by adding 0.05 ml of 12N H_2SO_4. Reaction products were extracted with ether. After removal of water with Na_2SO_4, ether was evaporated under N_2 stream. The residue was dissolved in a small amount of methanol, methylated with trimethylsilyldiazomethane (Tokyo Kasei), and subjected to GC–MS (JEOL JMS-SX102A equipped with Hewlett–Packard 5890, J&W DB5 column).

Table 1 shows the observed intensities of unlabeled and labeled resveratrol trimethyl ether at *m*/*z* 270–273. The labeled sample showed significant increase of intensities at *m*/*z* 271–273 due to deuterium incorporation. These increases can be resolved into individual isotopic spe-

Table 1. Mass spectral analysis of the isotopic composition of resveratrol trimethyl ether produced from deuterium-labeled malonyl–CoA based on the molecular ion at *m*/*z* 270

	Relative intensity (%)					
	Unlabeled resveratrol trimethyl ether	Resveratrol trimethyl ether from D_2 malonyl–CoA				
			Resolved into isotopic species			
m/*z*	Obsd.	Obsd.	M	M+D	M+2D	M+3D
270	100	100	100			
271	18.17	69.4	18.17	51.23		
272	1.46	19.6	1.46	9.31	8.83	
273	0	2.87		0.75	1.60	0.52

cies, as indicated in Table 1. From this isotopic resolution, the isotopomer composition (M:M+D:M+2D:M+3D = 62.3:31.9:5.50:0.32) was obtained, as shown in Table 2. The absolute value of 0.32% for M+3D species seems unreliable, but almost the same incorporation ratio was observed in two additional independent experiments (data not shown). This low incorporation rate must be due to deuterium–proton exchange at malonyl–CoA stage in the solution.

Not only the presence of isotopomer labeled with three deuterium atoms, but also the ratio of isotopomers could distinguish mechanism A from the other mechanisms (B, B′ and B″). Partially exchanged [D_2]–malony–CoA does not give the same composition of isotopomers of resveratrol in mechanism A and Bs, since the number of theoretically possible positions of deuterium incorporation differs between mechanism A and Bs. As shown in Fig. 3, the one-deuterium labeled species consists of two isotopomers (**a** and **b**) in mechanism A, but three isotopomers (**a**, **b** and **c**) in mechanism Bs. In addition, two deuterium-labeled resveratrol consists of only one isotopomer (**d**) in mechanism A, but of three (**d**, **e** and **f**) in mechanism Bs. Moreover, the probability of the presence of each isotopic species can be predicted by the quadratic form of deuterium retention (and/or no retention) per labeled site in mechanism A and by the cubic form in mechanism Bs, since there are two labeled positions in mechanism A, and three in mechanism Bs. Therefore, from the observed ratio of no deuterium species (62.3%), the percentage for deuterium retention per labeled site becomes 21.1% ($1-(0.623)^{1/2}$) in mechanism A, and 14.6% ($1-(0.623)^{1/3}$) in mechanism Bs. From these values, the ratio of isotopic species, $M:M+D:M+2D = (1-0.211)^2:2\times0.211\times(1-0.211):0.211^2 = 62.3:33.3:4.45$ in mechanism A and $M:M+D:M+2D:M+3D = (1-0.146)^3:3\times(1-0.146)^2\times0.146:3\times(1-0.146)\times0.146^2:0.146^3 = 62.3:31.9:5.46:0.31$ in mechanism Bs could be deduced. In Table 2, these ratios were compared with the observed isotopic compositions. It is apparent that the observed values match the calculated ones for mechanism Bs much more closely than those for mechanism A. These analyses of deuterium-labeled resveratrol thus exclude the intermediacy of stilbene–carboxylic acid, i.e. mechanism A.

Table 2. Observed and calculated ratio of isotopic species from partially exchanged deuterium-labeled malonyl–CoA

	Composition (%)			
	M	M+D	M+2D	M+3D
Observed[a]	62.3	31.9	5.50	0.32
Calcd for mechanism A[b]	62.3	33.3	4.45	0
Calcd for mechanism B, B′ and B″	62.3	31.9	5.46	0.31

[a] When the sum of isotopic species is set as 100%, the ratio of 100:51.23:8.83:0.52 in Table 1 becomes 62.3:31.9:5.50:0.32.

[b] Deuterium retention per labeled site is 21.1% in mechanism A, and 14.6% in mechanism B, B′ and B″.

As mentioned above, it is very difficult to distinguish three mechanisms (Bs) experimentally. But mechanism B is definitely different from mechanism B′ and B″ in the point that CoA thioester is hydrolyzed prior to cyclization. In both mechanism B′ and B″, cyclization of tetraketide takes place first before aromatization. Suppose either of mechanism B′ or B″ is operating, and spontaneous aromatization happens after cyclization (either at CoA bound or free stage) as a side-reaction (it is highly possible), then stilbenecarboxylic acid would be produced as a derailed product. However, all our previous attempts to detect stilbenecarboxylic in in vitro STS reaction were unsuccessful. On the other hand, presence of CTAL (the lactone form of *p*-coumaroyltriacetic acid, a hydrolysis product of tetraketide–CoA intermediate), in the STS reaction mixture, was rigorously established by LC–MS analysis.[3] As a by-product, CTAL was detected not only in the STS reaction but also in the CHS reaction, and its proportion was much larger in the total CHS products than in the STS products. This observation can be reasonably explained as follows. Free *p*-coumaroyltriacetic acid itself is an actual intermediate in the STS reaction, and is efficiently converted into resveratrol, but it is not a bona fide intermediate in the CHS reaction since the thioester must not be hydrolyzed before cyclization, as CoA–thiol anion must serve as a leaving group for Claisen condensation to release chalcone. *p*-Coumaroyltriacetic acid is a derailment product in the CHS

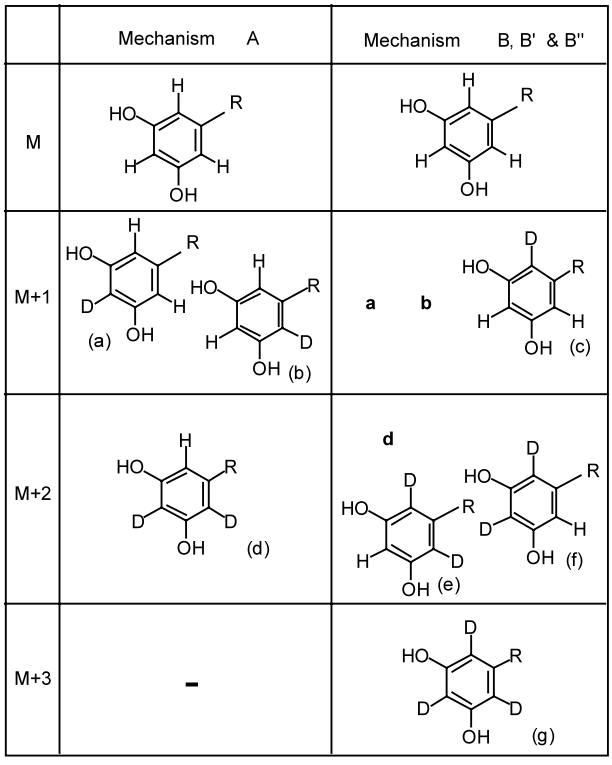

Figure 3. Isotopomers of resveratrol predicted to arise from partially exchanged [D_2]–malonyl–CoA in mechanisms A, B, B′ and B″.

reaction and remained without further reaction in the reaction mixture. For this reason, larger quantity of CTAL is always detected in the CHS reaction than in the STS reaction. If *p*-coumaroyltriacetic acid is not involved as an intermediate in the reaction of stilbene skeleton formation, CTAL must be released during condensation which is common to both CHS and STS reactions, and thus it should be produced in the same proportion in the both reactions. Although mechanisms B′ and B″ can not be excluded, these circumstantial facts suggest mechanism B as the most probable one among mechanism Bs.

The results of the present study demonstrated that resveratrol can be labeled with three deuterium atoms from deuterated malonyl-CoA in the STS reaction. This proves that stilbenecarboxylic acid is not an essential intermediate in peanut STS reaction. Exclusion of stilbenecarboxylic acid as an intermediate, together with the fact that larger quantity of CTAL is produced in STS reaction than in CHS reaction, strongly suggests that peanut STS first hydrolyzes the thioester of the tetraketide intermediate before cyclization and then carries out decarboxylative cyclization (mechanism B) to produce resveratrol. Stilbenecarboxylic acid-type compounds found in plants such as *Hydrangea macropylla*,[9] must be biosynthesized by specific enzymes via mechanisms quite different from that of peanut STS. The known CHS and STS share more than 60% identities in their amino acid sequences.[10] Although hydrolysis of CoA thioester and decarboxylation of intermediate are two unique features associated only with the STS reaction, no apparent motives for thioesterase and decarboxylase have been found in the known STS sequences. Recently, X-ray analysis of crystal structures of *Medicago sativa* CHS[11] has been carried out. In the active site, however, no functional amino acid residue for cyclization of tetraketide intermediate has been identified. It is extremely important to determine how these structurally similar enzymes control their different modes of cyclization, as they represent two major groups of the ever-increasing number of CHS superfamily members which elaborate the diverse structures of plant metabolites. Further studies to resolve this issue are underway.

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