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The biosynthesis of rosmarinic acid in suspension cultures of *Coleus blumei*

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Abstract

Suspension cultures of *Coleus blumei* accumulate very high amounts of rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxyphenyllactate, in medium with elevated sucrose concentrations. Since the synthesis of this high level of rosmarinic acid occurs in only five days of the culture period, the activities of the enzymes involved in the biosynthesis are very high. Therefore all the enzymes necessary for the formation of rosmarinic acid from the precursors phenylalanine and tyrosine could be isolated from cell cultures of *Coleus blumei*: phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase, hydroxycinnamoyl:CoA ligase, tyrosine aminotransferase, hydroxyphenylpyruvate reductase, rosmarinic acid synthase and two microsomal 3- and 3′-hydroxylases. The main characteristics of these enzymes of the proposed biosynthetic pathway of rosmarinic acid will be described.

Abbreviations: DHPL, 3,4-dihydroxyphenyllactate; DHPP, 3,4-dihydroxyphenylpyruvate; pHPL, 4-hydroxyphenyllactate, pHPP, 4-hydroxyphenylpyruvate; RA, rosmarinic acid

Introduction

Rosmarinic acid (RA) is one of the most commonly occurring caffeic acid esters in the plant kingdom. It is wide spread in the families Lamiaceae, mainly the subfamily Saturejoideae (Litvinenko et al. 1975; Mölgaard & Ravn 1988), and Boraginaceae, but can also be detected in other families, in ferns (Harborne 1966; Häusler et al. 1992) and in hornworts (Takeda et al. 1990; Zinsmeister et al. 1991; Petersen, unpublished). The molecular structure of rosmarinic acid as an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (Fig. 1) was clarified by Scarpati & Oriente (1958, 1960) using extracts of *Rosmarinus officinalis*.

The production of high amounts of RA by cell cultures of *Coleus blumei* was first described by Razzaque & Ellis (1977) and Zenk et al. (1977). These suspension cultures, together with cell cultures of *Anchusa officinalis*, were the main object for investigations on the biosynthesis of this phenolic compound. The precursors from primary metabolism for the biosynthesis of RA have been identified by Ellis & Towers (1970) by feeding radioactive amino acids to plants of *Mentha*. Phenylalanine was mainly incorporated into the caffeic acid part of the molecule, whereas tyrosine and DOPA gave rise to the 3,4-dihydroxyphenyllactic acid moiety. Essentially the same results were obtained with suspension cultures of *Coleus blumei* (Razzaque & Ellis 1977). Despite the knowledge of the biosynthetic precursors of RA, the first new enzyme of RA biosynthesis, tyrosine aminotransferase, was only described in 1987 by De-Eknamkul & Ellis (1987a, b). From this year on, the enzymes of RA biosynthesis successively became elucidated and a full biosynthetic pathway of RA in cell cultures of *Coleus blumei* has been proposed recently (Petersen et al. 1993).

Rosmarinic acid production in suspension cultures of *Coleus blumei*

The amount of RA accumulated by suspension cultures of *Coleus blumei* is highly dependent on the amount of carbohydrates added to the culture media (Zenk et

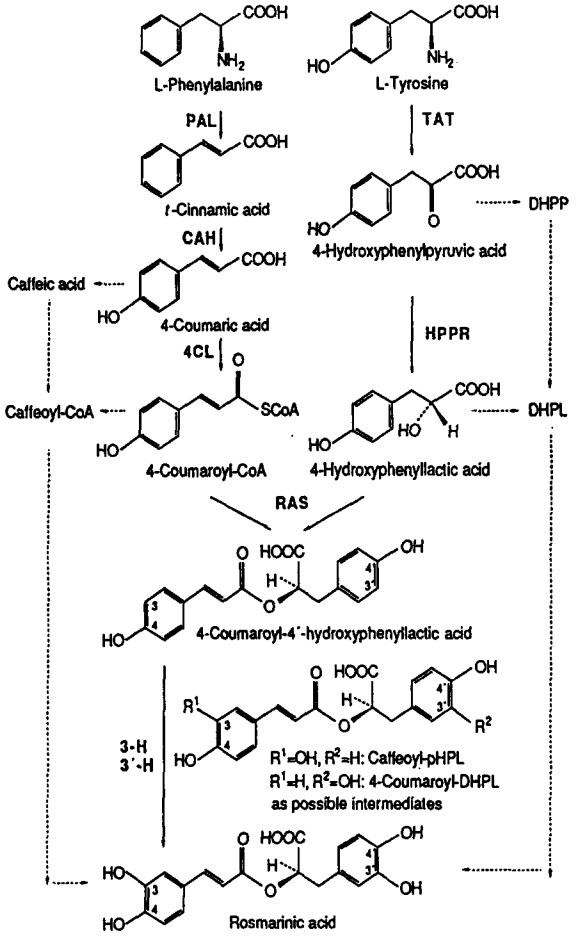

Fig. 1. Biosynthetic pathway for rosmarinic acid as deduced from the enzyme activities isolated from suspension cultures of *Coleus blumei*. PAL = phenylalanine ammonia-lyase; CAH = cinnamic acid 4-hydroxylase; 4CL = hydroxycinnamoyl:CoA ligase; TAT = tyrosine aminotransferase; HPPR = hydroxyphenylpyruvate reductase; RAS = rosmarinic acid synthase; 3H, 3'-H = 3- and 3'-hydroxylases.

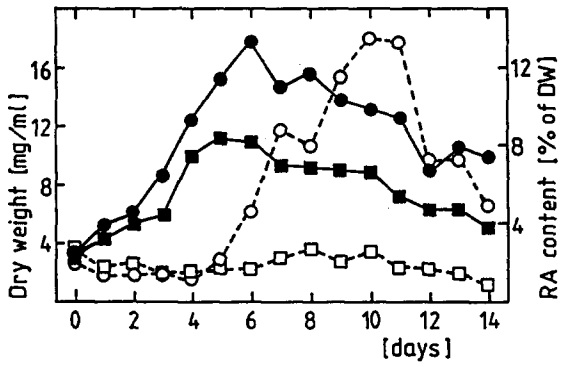

Fig. 2. Growth (depicted as dry weight accumulation: ■, ●) and rosmarinic acid accumulation (□, ○) of suspension cultures of *Coleus blumei* in media with 2% (■, □) and 4% sucrose (●, ○).

al. 1977; Ulbrich et al. 1985; Petersen & Alfermann 1988; Gertlowski & Petersen 1993). In our routinely used cultivation system, cells are maintained in a modified B5-medium (Gamborg et al. 1968; Petersen & Alfermann 1988) with 2% sucrose (CB2) where they only accumulate 2–3% of their dry weight as RA. Suspension cultures transferred to the same medium with 4% sucrose (CB4) produce up to 19% of their dry weight as RA at the end of the growth phase (Fig. 2). With higher sucrose concentrations even more RA can be obtained as long as the cells tolerate the osmotic pressure of the medium (Gertlowski & Petersen 1993). Although sucrose is cleaved into glucose and fructose, other sugars than sucrose support only lower RA accumulation.

The onset of net RA accumulation seems to be dependent on the limitation of an essential nutrient other than the carbon source. In the case of our *Coleus blumei* suspension cultures, the depletion of phosphate from the medium on day 4 to 5 of the culture period coincides with the start of RA synthesis and accumulation (Gertlowski & Petersen 1993). Reduction of the medium phosphate leads to a higher RA accumulation. Higher phosphate concentrations repress RA production in contrast to the results obtained for RA-producing cell cultures of *Anchusa officinalis* (De-Eknamkul & Ellis 1988). However, RA synthesis starts at about the same time as in media with lower phosphate levels showing that other nutrients may influence the onset of RA production as well.

Suspension cultures of *Coleus blumei* with their 'inducible' high RA accumulation have proven to be an excellent system for the investigation of the biosynthetic pathway of RA. A very high amount of this secondary compound is synthesized and accumulated in only about 5 days (from day 5 to 10) of the culture period resulting in high activities of the biosynthetic enzymes during this time.

The biosynthetic pathway of rosmarinic acid

A biosynthetic pathway for RA in suspension cultures of *Coleus blumei* derived from the enzyme activities isolated from these cells has been proposed recently (Petersen et al. 1993) and is depicted in Fig. 1. The enzyme activities involved in this pathway and their main characteristics (as far as already determined) are

Table 1. Some characteristics of the enzymes of rosmarinic acid biosynthesis in suspension cultures of *Coleus blumei*.

Enzyme	Localization	pH-optimum	Substrates	Other characteristics
Phenylalanine ammonia-lyase	soluble	7.8	phenylalanine K_m 0.16 μM	
Cinnamic acid 4-hydroxylase	membrane	7.5	cinnamic acid K_m 35 μM	cyt. P-450; inhibited by cyt. c;
			NADPH K_m 40 μM	dependent on O_2
Hydroxycinnamic acid:CoA ligase	soluble	7.5-8.0	4-coumarate K_m 2.2 μM	app. molecular mass
			(caffeate) K_m 5.0 μM	37 ± 2 kDa
			ATP K_m 73 (137) μM	
			coenzyme A K_m 3.0 (6.5) μM	
Tyrosine aminotransferase	soluble		tyrosine	dependent on pyridoxal-phosphate
			2-oxoglutarate	
Hydroxyphenylpyruvate reductase	soluble	6.5-7.0	pHPP K_m 10 μM	app. molecular mass
			DHPP K_m 130 μM	50-55 kDa; inhibited by
			NADPH K_m 95 μM	RA and 4-coumaroyl-CoA
			NADH K_m 190 μM	
Rosmarinic acid synthase	soluble	7.0-7.5	4-coumaroyl-CoA K_m 20 μM	inhibited by
			caffeoyl-CoA K_m 33 μM	* CoA (noncompetitively)
			±-pHPL K_m 0.17 mM	
			R(+)-DHPL K_m 0.37 mM	* *S*(-)-DHPL/* RA
reverse RAS reaction			RA K_m 15 μM	App. molecular mass
			CoA K_m 310 μM	77 KDa
3-Hydroxylase	membrane	7.5	4-coumaroyl-3′,4′-DHPL	inhibited by cyt. c;
			4-coumaroyl-4′-pHPL	dependent on O_2
			NADPH K_m 30 μM	
3′-Hydroxylase	membrane	7.5	caffeoyl-4′-pHPL	inhibited by cyt. c;
			4-coumaroyl-4′-pHPL	dependent on O_2
			NADPH K_m 30 μM	

summarized in Table 1 and described in more detail in the following paragraphs.

Phenylalanine-derived pathway

Phenylalanine as the precursor for the caffeic acid moiety of RA (Ellis & Towers 1970; Razzaque & Ellis 1977) as well as the correlation of the activity of phenylalanine ammonia-lyase (PAL) with RA synthesis (Razzaque & Ellis 1977) suggested the participation of the general phenylpropanoid metabolism in the biosynthesis of RA. The respective enzymes, PAL, cinnamic acid 4-hydroxylase (CAH) and hydroxycinnamic acid:CoA ligase (4CL), have been shown to be correlated in their activities to RA synthesis and accumulation in cell cultures of *Coleus blumei* (Karwatzki et al. 1989).

Phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase (PAL) catalyzes the oxidative desamination of phenylalanine and forms t-cinnamic acid and ammonia. The enzyme from suspension cultures of *Coleus blumei* is a soluble enzyme and precipitates between 40 and 55% saturation of ammonium sulphate. The affinity of the enzyme towards its substrate phenylalanine is very high with an apparent K_m-value of about 16 μM. RA up to 1 mM does not inhibit PAL activity. Highest activity in cultures in medium with 4% sucrose is found on day 6 of the culture period, 4 days earlier than the maximum RA accumulation (cf. Fig. 2).

Cinnamic acid 4-hydroxylase

Cinnamic acid 4-hydroxylase (CAH) introduces the para-hydroxyl group into the aromatic ring of t-cinnamic acid by a cytochrome P-450-dependent monooxygenation reaction. The enzyme activity essentially depends on molecular oxygen and NADPH.

CAH isolated from cell cultures of *Coleus blumei* in CB4-medium exerts its maximal activity around day 6 of the culture period as PAL. The microsomal enzyme has a pH-optimum of pH 7.5. CAH is not inhibited by phenolase inhibitors (diethyldithiocarbaminate, salicylhydroxamic acid) and potassium cyanide, but as low as 10 µM cytochrome c totally blocks the enzyme activity. This is a typical characteristic of cytochrome P-450-dependent enzymes, where cytochrome c acts as a competitive electron acceptor of NADPH:cytochrome P-450 reductase. FAD, a component of cytochrome P-450 reductase, stimulates the activity of CAH by more than 20%. NADPH is the only electron donor accepted by CAH; NADH is not used by CAH and no synergistic effect of NADH and NADPH can be observed for the enzyme from *Coleus blumei* in contrast to CAH from some other plants (e.g., Benveniste et al. 1977, 1982). The reaction was saturated at NADPH concentrations of 0.5 mM and a K_m-value of around 40 µM was determined for this substrate. The saturation concentration of CAH for cinnamic acid is at 1 mM with a K_m-value at 35 µM.

Activation of cinnamic acids

For the formation of cinnamic acid esters usually an activated cinnamic acid is essential. The activation energy can be achieved by different activation modes: the cinnamic acid can be activated as coenzyme A-thioester (e.g., flavonoid biosynthesis (Kreuzaler & Hahlbrock 1972), chlorogenic acid biosynthesis (Stöckigt & Zenk 1974)), as cinnamic acid glucose ester (e.g., biosyntheses of sinapoylmalate (Tkotz & Strack 1980) or chlorogenic acid (Kojima & Villegas 1984)) or as another cinnamic acid ester, such as chlorogenic acid (e.g., biosyntheses of dicaffeoyl quinic acid (Villegas et al. 1987) or caffeoyl glucaric acid (Strack et al.1987)). For RA biosynthesis cinnamic acid CoA-esters proved to be the only accepted activation form (Petersen 1991).

Hydroxycinnamoyl:CoA ligase

Hydroxycinnamoyl:CoA-ligase (4CL) catalyzes the activation of cinnamic acids with coenzyme A in an ATP-dependent reaction. Like PAL and CAH the enzyme from cell cultures of *Coleus blumei* in CB4-medium is most active at day 6 of the culture period. The soluble enzyme precipitates between 30 and 55% saturation of ammonium sulphate and has a pH-optimum of 7.5–8.0. The enzyme was partially purified by different purification methods, however, isoenzymes could not be resolved by these methods. The apparent molecular mass of 4CL from suspension cultures of *Coleus blumei* was at 37 ± 2 kDa. The kinetic characteristics of 4CL were determined using partially purified enzyme preparations (Karwatzki 1992). Several cinnamic acids (cinnamic, 4-coumaric, caffeic and ferulic acid) were accepted as substrates by the enzyme from *Coleus blumei*. 4-Coumarate was the best substrate with a K_m-value of 2.2 µM, the K_m value for caffeic acid was determined at 5.0 µM. Slightly different K_m-values could be observed for ATP and coenzyme A using either 4-coumarate or caffeate as substrates: the K_m-values for ATP with 4-coumarate and caffeate as substrates were at 73 µM and 137 µM, respectively and for coenzyme A at 3.0 µM and 6.5 µM, respectively (Karwatzki 1992). However, up to now different isoenzymes of hydroxycinnamoyl:CoA transferase could not be resolved.

With 4-coumaroyl-CoA as the direct precursor for the ester formation in the biosynthetic pathway of RA the final intermediate of the phenylalanine-derived pathway is reached.

Tyrosine-derived pathway

Feeding experiments with radioactive amino acids resulted in the identification of tyrosine and DOPA as precursors for the 3,4-dihydroxyphenyllactic acid moiety of RA (Ellis & Towers 1970; Razzaque & Ellis 1977). In 1987, tyrosine aminotransferase was identified as the first enzyme of the tyrosine-derived pathway of RA biosynthesis, whereas the involvement of tyrosine 3-hydroxylase and tyrosine oxidase were found to be unlikely (Ellis et al.1979; De-Eknamkul & Ellis 1987a). Therefore DOPA did no longer seem to be a direct precursor for RA.

Tyrosine aminotransferase

Tyrosine aminotransferase (TAT) catalyzes the transamination of tyrosine with help of 2-oxoglutarate giving rise to 4-hydroxyphenylpyruvate and glutamate. This pyridoxal phosphate-containing enzyme has been purified and characterized from RA synthesizing cell cultures of *Anchusa officinalis* (De-Eknamkul & Ellis 1987a, b; Mizukami & Ellis 1991). The activity of TAT is induced in cell cultures of *Coleus blumei* (from CB4-medium) together with the enzymes of the general phenylpropanoid metabolism in a coordinated way showing maximal activity at day 6 of the culture period. Since this enzyme is well characterized from cell

cultures of *Anchusa officinalis* (De-Eknamkul & Ellis 1987a, b; Mizukami & Ellis 1991) a detailed characterization of the enzyme from *Coleus blumei* has not yet been undertaken.

Hydroxyphenylpyruvate reductase

Hydroxyphenylpyruvate reductase (HPPR) reduces 4-hydroxyphenylpyruvate (pHPP) to 4-hydroxyphenyllactate (pHPL) with help of NAD(P)H (Petersen & Alfermann 1988; Häusler et al. 1991; Meinhard et al. 1992). This enzyme was first isolated and characterized from cell cultures of *Coleus blumei* where it shows its maximum activity in cell extracts from CB4-medium at day 6 of the culture period.

In a number of cases the reduction of aromatic 2-oxoacids has been described as secondary function of malate or lactate dehydrogenase (Friedrich et al. 1987, 1988). However, the enzyme from *Coleus blumei* could be proven to be a specific hydroxyphenylpyruvate reductase (Meinhard, unpublished results). Only a NADP-dependent, but no NAD-dependent oxidation of malate was detectable in enzyme extracts from *Coleus blumei*, whereas the reduction of hydroxyphenylpyruvate by HPPR uses both, NADH and NADPH. A slight and rapidly stagnating oxidation of lactate could be attributed to the activity of HPPR. Furthermore, oxamate, a specific inhibitor for lactate dehydrogenase, did not influence the activity of HPPR.

HPPR from cell cultures of *Coleus blumei* is a soluble enzyme which was partially purified by fractionated ammonium sulphate precipitation (40–60% saturation), hydroxylapatite adsorption chromatography and affinity chromatography on 2',5'-ADP-Sepharose 4B (Meinhard et al. 1992). The apparent molecular mass of this enzyme is 50–55 kDa. HPPR has a pH-optimum of pH 6.5–7.0. Besides pHPP also other hydroxyphenylpyruvates are accepted as substrates as long as they provide a free 4-hydroxyl group in the aromatic ring.

Therefore phenylpyruvate was not reduced. The K_m values for pHPP and 3,4-dihydroxyphenylpyruvate (DHPP) were determined at 10 µM and 130 µM respectively, suggesting that pHPP might be the natural substrate for HPPR (Häusler et al. 1991). Both NADH and NADPH were accepted as reduction equivalents by HPPR, but the higher affinity was determined for NADPH with a K_m value of 95 µM in contrast to 190 µM for NADH. The reaction velocities, however, were higher for NADH. Probably the enzyme can accept both electron donors in its natural environment. The activity of HPPR is inhibited by 4-coumaroyl-CoA and RA, whereas other cinnamic acids or cinnamic acid CoA-esters had no influence on HPPR activity. This inhibition by the end product of the biosynthetic pathway and the final intermediate of the phenylalanine-derived pathway could indicate a possible regulatory function of HPPR. Other inhibitors of HPPR were pyruvate and oxaloacetate at concentrations of 4 mM and 5 mM respectively. HPPR also catalyzes the reverse reaction, the oxidation of hydroxyphenyllactates with help of NAD(P).

The reaction product of HPPR, 4-hydroxyphenyllactate, is the immediate precursor for the formation of the hydroxycinnamoyl ester.

Ester formation: Rosmarinic acid synthase

The enzyme catalyzing the formation of the ester from the two precursors synthesized from phenylalanine and tyrosine is rosmarinic acid synthase (RAS) which transfers the hydroxycinnamoyl moiety from hydroxycinnamoyl-CoA to the aliphatic hydroxyl group of hydroxyphenyllactate. This enzyme was first isolated and described from cell cultures of *Coleus blumei* (Petersen & Alfermann 1988). The natural substrates of this enzyme presumably are 4-coumaroyl-CoA and 4-hydroxyphenyllactate resulting in the first ester 4-coumaroyl-4'-hydroxyphenyllactate (pC-pHPL). Therefore the systematic name of rosmarinic acid synthase (RAS) would be 4-coumaroyl-CoA:4-hydroxyphenyllactate 4-coumaroyl-transferase (Petersen 1991). Maximum activity of this enzyme during a culture period of suspension cultures of *Coleus blumei* in CB4-medium is obtained at day 7–8, somewhat later than the activities of the enzymes providing the substrates for the RAS reaction. RAS accepts a number of substrates giving rise to a variety of esters some of which have not been described to occur in nature up to now. The structures of the esters formed from 4-coumaroyl- and caffeoyl-CoA and 4-hydroxy- and 3,4-dihydroxyphenyllactate have been elucidated by ion spray mass spectrometry and tandem mass spectrometry (Petersen & Metzger 1993). Cinnamoyl-, 4-coumaroyl- and caffeoyl-CoA are accepted by RAS (the yield of the reaction products with sinapoyl- and feruloyl-CoA did not allow a further analysis). The highest affinity was determined for 4-coumaroyl-CoA with a K_m-value around 20 µM, the enzyme showed a strong substrate inhibition by this substrate. Caffeoyl-CoA was accepted with a K_m value of 33 µM and the enzyme was saturated at a concentration of 100

μM. Competition experiments with the simultaneous application of varying concentrations of 4-coumaroyl- and caffeoyl-CoA also indicated that 4-coumaroyl-CoA is the favoured substrate of RAS. Essential for the other substrate of RAS is a free 4-hydroxyl group at the aromatic ring and the R(+)-configuration of the hydroxyphenyllactate. Therefore 4-hydroxy-, 3,4-dihydroxy- and 3-methoxy-4-hydroxyphenyllactate were accepted, whereas phenyllactate and 3,4-dimethoxyphenyllactate did not serve as substrates. RAS was saturated at 1 mM of 4-hydroxy- and 3,4-dihydroxyphenyllactate (pHPL, DHPL) and K_m-values of 0.17 mM and 0.37 mM respectively, were determined. For these experiments, however, only the racemic mixture of pHPL could be used, whereas pure R(+)-DHPL was available. Since S(-)-DHPL strongly inhibited the RAS reaction, the true K_m-value for pHPL (R(+)) might even be lower. The catalytic acitivity of RAS is inhibited by pHPP and DHPP and the S(-)-isomer of DHPL, but not by cinnamic acids. Coenzyme A, however, is a strong inhibitor of the ester formation. The mechanism of this inhibition is non-competitive with inhibition constants of K_i=K_{ii}=1.7 mM. RAS catalyzes the reverse reaction, the splitting of rosmarinic acid in presence of coenzyme A into caffeoyl-CoA and 3,4-dihydroxyphenyllactate as well. This reaction needs a concentration of 50 μM of RA for saturation (K_m 15 μM), but the saturation concentration for coenzyme A is rather high at 1 mM (K_m 310 μM) indicating that this reaction might not be important *in vivo*.

3- and 3'-Hydroxylations of the aromatic rings

For a considerable time it was not clear whether RA is directly formed from caffeoyl-CoA and 3,4-dihydroxyphenyllactate or whether the introduction of the 3-hydroxyl groups of the aromatic rings occurs at a later stage of the biosynthetic pathway. Specific enzymes catalyzing the 3-hydroxylation of 4-coumaric acid or 4-coumaroyl-CoA have been described in a few cases (Kamsteeg et al. 1981; Boniwell & Butt 1986; Kneusel et al. 1989; Kojima & Takeuchi 1989), but no such enzymes are known from plants for hydroxyphenylpyruvic or –lactic acids besides phenolases, which are ubiquitous and commonly not regarded as specific (Butt 1985). From suspension cultures of *Coleus blumei* specific hydroxylases introducing the 3- and 3'-hydroxyl groups into rosmarinic acid-like esters (4-coumaroyl-4'-hydroxyphenyllactic acid, 4-coumaroyl-3',4'-dihydroxyphenyllactic acid, caffeoyl-4'-hydroxyphenyllactic acid) could be detected in microsomal preparations (Petersen et al. 1993). Monophenolic substrates like 4-coumarate, 4-coumaroyl-CoA, 4-hydroxyphenylpyruvate or 4-hydroxyphenyllactate were not accepted by these hydroxylases. Because of this strong specificity for RA-like esters it was suggested that in RA biosynthesis the introduction of the 3- and 3'-hydroxyl groups are the last two reactions of the biosynthetic pathway. These hydroxylases have a number of common characteristics with cinnamic acid 4-hydroxylase and therefore might also be cytochrome P-450-dependent monooxygenases. They require molecular oxygen and NADPH for a successful hydroxylation reaction and are strongly inhibited by cytochrome c (10 μM for a 100% inhibition), a competitive inhibitor of NADPH:cytochrome P-450 reductase. On the other hand, phenolase inhibitors (diethyldithiocarbaminate, salicylhydroxamic acid) or potassium cyanide do not negatively influence the hydroxylations. NADH can only sustain around 10% of the hydroxylation activities obtained with NADPH as electron donor. FAD, which is a component of NADPH:cytochrome P-450 reductase, stimulates the enzyme activities by 20%. Both, 3- and 3'-hydroxylase activities, were saturated with 0.5 mM NADPH and showed an apparent K_m-value of 30 μM for this substrate. Up to now it remains unclear whether those two hydroxylation reactions are catalyzed by the same enzyme or two independent enzymes. Inhibition studies with cytochrome P-450-inhibitors, however, indicate the participation of two differently sensitive enzyme systems (Petersen, unpublished results). The participation of cytochrome P-450 in the 3- and 3'-hydroxylations of RA-like esters still remains to be proven.

With the final introduction of the 3- and 3'-hydroxyl groups of the ester the end product of the biosynthetic pathway, rosmarinic acid, is formed.

Discussion and conclusions

With help of the high-producing cell suspension cultures of *Coleus blumei* and *Anchusa officinalis* the biosynthetic pathway of rosmarinic acid has been elucidated (De-Eknamkul & Ellis 1987a, b; Petersen & Alfermann 1988; Petersen et al.1993). All enzyme activities necessary for the formation of RA from the precursors phenylalanine and tyrosine (Ellis & Towers 1970) could be isolated from cell cultures of *Coleus blumei* grown in a medium with 4% sucrose which strongly enhances RA production. Since the formation of up to 20% of the cell dry weight as RA is accom-

plished in only about 5 days of the culture period, the activities of the involved enzymes must be very high. This is in contrast to whole plants where often lower secondary product concentrations are synthesized over a long period. This shows again the usefulness of plant cell cultures for biosynthetic studies of secondary pathways as described by Zenk (1991).

In RA biosynthesis, phenylalanine is transformed into 4-coumaroyl-CoA by the enzymes of the general phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (CAH) and hydroxycinnamoyl:CoA ligase (4CL) resulting in 4-coumaryol-CoA as the activated precursor for the ester formation. 4-Hydroxyphenyllactate is formed from tyrosine by the activities of tyrosine aminotransferase (TAT) and hydroxyphenylpyruvate reductase (HPPR). These two enzymes have been newly detected and described as enzymes of secondary metabolism (De-Eknamkul & Ellis 1987a; Petersen & Alfermann 1988). Their substrate specificities and the induction of their activities in correlation to RA biosynthesis can be taken as evidences that TAT and HPPR are specific enzymes for this secondary metabolic pathway although the same or similar enzymes are also involved in primary pathways. The formation of the presumably first ester, 4-coumaroyl-4'-hydroxyphenyllactate, is catalyzed by rosmarinic acid synthase (RAS) under the release of coenzyme A (Petersen & Alfermann 1988; Petersen 1991). This enzyme as well as 4CL and HPPR are not specific with regard to the hydroxylation pattern of the aromatic rings of the respective substrates. Therefore the question was whether the 3-hydroxyl groups of both aromatic rings are introduced prior to or after the formation of the ester. Specific 3-hydroxylases for 4-coumaric acid or 4-coumaroyl-CoA have been described only for a few cases and the respective enzymes, with exception of the ubiquitous phenolases, do not seem to occur frequently. The identification of hydroxylase activities in microsomal preparations from cell cultures of *Coleus blumei* which are able to introduce the 3- and 3'-hydroxyl groups specifically into the aromatic rings of RA-like esters sustains the assumption that the 3- and 3'-hydroxylation reactions are the final biosynthetic steps in RA biosynthesis. This corresponds to findings in the biosynthetic pathways of flavonoids and chlorogenic acid where the introduction of the hydroxylation patterns of the aromatic rings occurs after the completion of the C-skeleton as well (Forkmann et al. 1980; Stotz & Forkmann 1982; Hagmann et al. 1983; Heller & Kühnl 1985; Larson & Bussard 1986; Kühnl et al. 1987). In a strict sense, the above described biosynthetic pathway of RA is only proven for cell cultures of *Coleus blumei*. The validity of this scheme has to be shown for other systems and whole plants as well. Some of the respective enzymes have already been found to be active in plants of *Coleus blumei* (Szabo, unpublished results). An interesting task will be to demonstrate whether the same biosynthetic pathway of RA is used in evolutionary distant plants as ferns and hornworts.

The identification of all the enzymes necessary for RA biosynthesis now enables us to investigate the regulation of this biosynthetic pathway. Under conditions which allow a high production of RA (high sugar content of the culture medium) the activities of the biosynthetic enzymes are coordinately stimulated (Karwatzki et al. 1989). The enzymes catalyzing the formation of the precursors for the ester all have their maximum activities on day 6 of the culture period, whereas the ester forming enzyme (RAS) is most active on day 7–8 and maximal RA accumulation is observed on day 10 of the culture period. In media with low sugar concentrations the activities of all the enzymes as well as RA accumulation remain low. This system is suitable for investigations on the regulation of this secondary pathway since only a limited number of enzymes is involved, but the intermediates are formed in coordinately regulated parallel pathways. For this purpose a number of enzymes of RA biosynthesis have already been purified and first experiments for the cloning of genes coding for RA biosynthetic enzymes have already been performed. Further investigations will hopefully show us whether RA biosynthesis is regulated by similar mechanisms as described for example for anthocyanin formation (e.g., Lloyd et al. 1992).

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