

# Two New Enzymes of Rosmarinic Acid Biosynthesis from Cell Cultures of *Coleus blumei*: Hydroxyphenylpyruvate Reductase and Rosmarinic Acid Synthase

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Rosmarinic acid biosynthesis can be stimulated in cell cultures of *Coleus blumei* by culturing the cells in medium with 4% sucrose. In enzyme extracts of these cells two new enzymes of rosmarinic acid biosynthesis were discovered. Hydroxyphenylpyruvate reductase reduces 4-hydroxyphenylpyruvate and 3,4-dihydroxyphenylpyruvate to 4-hydroxyphenyllactate and 3,4-dihydroxyphenyllactate, respectively, using NADH. Rosmarinic acid synthase transfers the caffeoyl moiety of caffeoyl-CoA to the non-phenolic OH-group of 3,4-dihydroxyphenyllactic acid, in course of which rosmarinic acid is formed.

## Introduction

Rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, is an abundant secondary plant product in species of the Lamiaceae and Boraginaceae. It was first isolated by Scarpati and Oriente from *Rosmarinus officinalis* [1]. Rosmarinic acid is of pharmaceutical interest because of its anti-complement, anti-oxidant and anti-inflammatory action [2]. Cell cultures of *Anchusa officinalis* and *Coleus blumei* were shown to produce high amounts of rosmarinic acid [4, 5, 7]. The influence of culture conditions, sucrose content, macronutrients and plant hormones on the biosynthetic activity of the cell cultures were investigated [4, 7–9]. Especially, high sucrose levels in the culture medium were able to rise the content of rosmarinic acid up to 15% of the dry weight of the cells [7]. According to feeding experiments of radioactive precursors to *Mentha* plants and cell cultures of *Anchusa officinalis* and *Coleus blumei* the caffeoyl moiety of rosmarinic acid is derived from phenylalanine, the 3,4-dihydroxyphenyllactic acid part from tyrosine or DOPA [3–5]. The two parallel pathways leading to the precursors of rosmarinic acid seem to be tightly coupled [6]. In contrast to the numerous publications on the produc-

tion of rosmarinic acid by cell cultures, only two enzymes involved in rosmarinic acid biosynthesis were described up to now. Phenylalanine ammonia lyase, the key enzyme for the synthesis of caffeic acid was shown to be active in cell cultures of *Coleus blumei* [5, 6]. Tyrosine aminotransferase, the first enzyme in the biosynthesis of the 3,4-dihydroxyphenyllactic acid moiety of rosmarinic acid was found in cell cultures of *Coleus blumei* and *Anchusa officinalis* [10, 11].

We now report on two new enzymes of the rosmarinic acid biosynthetic pathway, a hydroxyphenylpyruvic acid reductase, which catalyzes the reduction of 4-hydroxyphenylpyruvic acid or 3,4-dihydroxyphenylpyruvic acid to the corresponding lactic acids and rosmarinic acid synthase (caffeoyl-Coenzyme A: 3,4-dihydroxyphenyllactic acid caffeoyl transferase), the enzyme transferring the caffeoyl moiety from caffeoyl-CoA to 3,4-dihydroxyphenyllactic acid. This is the crucial enzyme in rosmarinic acid biosynthesis forming the ester linkage between the caffeic acid moiety and the 3,4-dihydroxyphenyllactic acid moiety.

## Materials and Methods

### Cell suspension cultures

Suspension cultures of *Coleus blumei* were a gift from Dr. Ulbrich (A. Nattermann & Cie. GmbH, Cologne, F.R.G.). 20 ml of the cell suspension was subcultivated every 7 days in 50 ml of a modified B5-medium [12] containing 0.5 mg/l IAA, 2 mg/l

**Abbreviations:** RA, rosmarinic acid; DHPL, 3,4-dihydroxyphenyllactic acid; DTT, dithiothreitol.

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2,4-D, 0.5 mg/l NAA, 0.2 mg/l kinetin, 2 g/l NZ-Amine (Otto Aldag, Hamburg, F.R.G.) and 2% sucrose on a gyratory shaker (120 rpm, 5 cm stroke) at 25 °C in the dark. For induction of rosmarinic acid biosynthesis the same medium as above, but containing 4% sucrose, was used.

### Chemicals

Caffeic acid and *p*-coumaric acid were purchased from Merck (Darmstadt, F.R.G.), Coenzyme A (free acid) and NADH from Fluka (Neu-Ulm, F.R.G.). The CoA-esters of caffeic and *p*-coumaric acid were synthesized *via* succinimid esters according to [13]. *p*-Hydroxyphenylpyruvic acid was obtained from Serva (Heidelberg, F.R.G.). 3,4-Dihydroxyphenylpyruvic acid was a gift from Dr. N. Rao (KFA Jülich, F.R.G.). 3,4-Dihydroxyphenyllactic acid was prepared by enzymatic hydrolysis of rosmarinic acid with Rhozyme HP-150 (Pollock and Pool Ltd., Reading, Great Britain) in 0.1 M ammonium acetate buffer pH 6.5 at 30 °C for 4 h. The 3,4-dihydroxyphenyllactic acid was extracted with ethylacetate and was purified by TLC on silica gel in solvent system II. The concentration of 3,4-dihydroxyphenyllactic acid in aqueous solution was determined spectrophotometrically at 280 nm using an extinction coefficient of  $2600 \text{ M}^{-1}\text{cm}^{-1}$  [5]. Rosmarinic acid was a gift from Dr. Ulbrich (as above).

### Preparation of enzyme extracts

7 day old suspension cultures of *Coleus blumei* grown in medium with 4% sucrose were filtered under suction. The following preparation was performed at 4 °C. The cells were homogenized with an Ultra-Turrax (Janke und Kunkel, Staufen i.Br., F.R.G.) for 90 seconds in 0.5 ml buffer (0.1 M  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ , 1 mM DTT, pH 7.0) per g fresh weight of cells together with 10% Polyclar AT (Serva, Heidelberg, F.R.G.). The homogenate was filtered through Miracloth and centrifuged at  $48,000 \times g$  for 15 min. The supernatant was fractionated with  $(\text{NH}_4)_2\text{SO}_4$ . The hydroxyphenylpyruvate reductase was precipitated between 50 and 80% saturation of  $(\text{NH}_4)_2\text{SO}_4$ , the rosmarinic acid synthase between 60 and 80%. The precipitated protein was sedimented at  $48,000 \times g$  for 20 min, redissolved in buffer and desalted on a Sephadex G-25-column (Pharmacia, Uppsala, Sweden). The eluting protein was

used for enzyme assays. The enzyme preparation could be stored at  $-18^\circ\text{C}$  for several weeks.

### Assay for hydroxyphenylpyruvic acid reductase

Standard reductase assays contained in a final volume of 0.25 ml of buffer (as above): 0.5  $\mu\text{mol}$  3,4-dihydroxyphenylpyruvic acid, 25 nmol ascorbate, 0.5  $\mu\text{mol}$  NADH, 0.25  $\mu\text{mol}$  DTT and 10–25  $\mu\text{l}$  of desalted enzyme preparation.  $\text{N}_2$  was bubbled through the reaction vessel before closing it. The reaction proceeded for 10 minutes at 30 °C in the dark. It was stopped by adding 10  $\mu\text{l}$  of 6 N HCl. The reaction product was extracted twice with 0.5 ml of ethylacetate. After evaporating the organic solvent under vacuum the residue was redissolved in 0.3 ml of 33% methanol/67% water adjusted to pH 3 with  $\text{H}_3\text{PO}_4$  (85%). The products were identified and quantified by HPLC using a Shandon Hypersil ODS column (particle size 5  $\mu\text{m}$ , length 25 cm, diameter 4.6 mm) by isocratic elution with 20% methanol/80% water containing 100  $\mu\text{l}$   $\text{H}_3\text{PO}_4$  (85%) per l. The flow rate was 1.5 ml per minute. The products were detected spectrophotometrically at 280 nm.

### Assay for rosmarinic acid synthase

The assay contained in a total volume of 0.25 ml of buffer (as above): 125 nmol ascorbate, 2.5  $\mu\text{mol}$  DTT, 50 nmol caffeoyl-CoA, 100 nmol 3,4-dihydroxyphenyllactic acid and 10–25  $\mu\text{l}$  of desalted enzyme preparation. The reaction proceeded for 10 min at 30 °C and was terminated by adding 10  $\mu\text{l}$  of 6 N HCl. The rosmarinic acid was extracted twice with 0.5 ml of ethylacetate. The ethylacetate was evaporated under vacuum and the residue was redissolved in 300  $\mu\text{l}$  of 33% methanol/67% water adjusted to pH 3 with  $\text{H}_3\text{PO}_4$  (85%). Rosmarinic acid and other reaction products were quantified using HPLC as above but with the solvent methanol/water 1:1 containing 100  $\mu\text{l}$   $\text{H}_3\text{PO}_4$  (85%) per l. The reaction products were detected spectrophotometrically at 333 nm.

### Solvent systems used for TLC on silica gel plates

(I) toluol/ethylformiate/formic acid 5:4:1; (II) *n*-butanol/acetic acid/water 63:10:27.

### Protein determination

Protein concentrations were measured according to [14] using bovine serum albumin as a standard.



## Results and Discussion

### *Induction of rosmarinic acid biosynthesis in suspension cultures of Coleus blumei*

*Coleus* cells cultivated in modified B5-medium with 2% sucrose accumulate only low amounts of rosmarinic acid. The production of rosmarinic acid can be induced in medium containing 4% sucrose. The highest biosynthetic rate for rosmarinic acid can then be observed from day 6 to day 8 of the culture period, which coincides with the end of the logarithmic growth phase. At the end of the growth phase the rosmarinic acid content of the cell cultures is 19% of the dry weight of the cells.

In enzyme extracts of 7 day old induced *Coleus blumei* cells several enzymes involved in rosmarinic acid biosynthesis were detected. From the pathway leading from phenylalanine to caffeoyl-CoA or *p*-coumaroyl-CoA phenylalanine ammonia lyase, cinnamic acid 4-hydroxylase, caffeic acid and *p*-coumaric acid CoA-ligase and a phenolase transforming *p*-coumaric acid to caffeic acid were detected (Ellen Vetter, unpublished results). From the pathway leading from tyrosine to 3,4-dihydroxyphenyllactic acid tyrosine aminotransferase [10, 11] was also active in our protein preparations and a new enzyme, hydroxyphenylpyruvate reductase, was identified. Finally, we detected rosmarinic acid synthase, the enzyme forming the ester linkage between caffeic acid and 3,4-dihydroxyphenyllactic acid.

### *Hydroxyphenylpyruvic acid reductase*

The hydroxyphenylpyruvic acid reductase present in enzyme preparations from *Coleus blumei* cells reduces 4-hydroxyphenylpyruvic acid and 3,4-dihydroxyphenylpyruvic acid to the corresponding lactic acids. The reductase is a soluble enzyme, which can be precipitated between 50 and 80% saturation of  $(\text{NH}_4)_2\text{SO}_4$ . The enzyme utilizes NADH as an electron donor, but also accepts NADPH with a lower activity. The enzyme is stimulated by DTT and ascorbic acid. Ascorbate probably prevents oxidation of the substrates as well as the products, which are quite sensitive to oxidation by light and oxygen. For the same reason the reaction has to be performed in the dark and under  $\text{N}_2$ . The reaction products were isolated by preparative TLC and were identified by comparison of spectrophotometrical data of authentic 4-hydroxyphenyllactic acid and of 3,4-dihydroxy-

phenyllactic acid prepared by hydrolysis of rosmarinic acid, respectively, as well as by co-chromatography on TLC on silica gel in solvent systems I and II and by HPLC (Fig. 1).

### *Rosmarinic acid synthase*

Rosmarinic acid synthase transfers the caffeoyl moiety of caffeoyl-CoA to the non-phenolic OH-group of 3,4-dihydroxyphenyllactic acid. The identity of the reaction product with rosmarinic acid was demonstrated by co-chromatography of authentic rosmarinic acid with the reaction product in HPLC (Fig. 2) and by TLC on silica gel in solvent systems I and II. Furthermore the reaction product was chromatographed one-dimensionally on silica gel plates in solvent system I, then the rosmarinic acid spot was hydrolyzed by treatment with Rhozyme and the hydrolysis products were separated by chromatography in the second dimension in the same solvent. The hydrolysis products showed the same behaviour as co-chromatographed caffeic acid and 3,4-dihydroxyphenyllactic acid. Rosmarinic acid synthase is a soluble enzyme, which precipitates be-

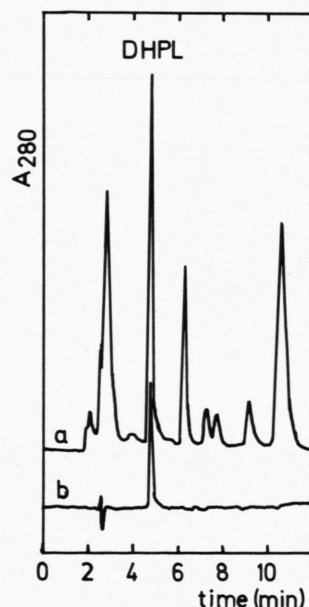


Fig. 1. HPLC chromatogram of the ethylacetate extract of a hydroxyphenylpyruvate reductase assay with 3,4-dihydroxyphenylpyruvate as substrate and 3,4-dihydroxyphenyllactic acid as product (a) and of 3,4-dihydroxyphenyllactic acid prepared by enzymatic hydrolysis of rosmarinic acid (b). For HPLC parameters see Materials and Methods.

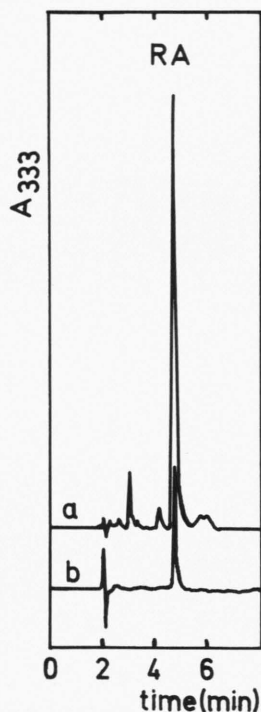


Fig. 2. HPLC chromatogram of the ethylacetate extract of a rosmarinic acid synthase assay with caffeoyl-CoA and 3,4-dihydroxyphenyllactic acid as substrates (a) and of authentic rosmarinic acid (b). For HPLC parameters see Materials and Methods.

tween 60 and 80% saturation of  $(\text{NH}_4)_2\text{SO}_4$ . The enzyme activity is enhanced by addition of SH-reagents, for example 10 mM DTT. A slight increase in enzyme activity is observed with addition of 0.5 mM ascorbic acid. This is probably due to stabilization of the oxygen sensitive substrates. Rosmarinic acid synthase can be stored at  $-18^\circ\text{C}$  for several weeks. No loss in activity can be observed after incubation of the enzyme at  $30^\circ\text{C}$  for 5 h. The enzyme is not restricted to caffeoyl-CoA as the cinnamoyl moiety

and also accepts 4-hydroxyphenyllactic acid besides 3,4-dihydroxyphenyllactic acid as cinnamoyl acceptor. Therefore several other reaction products could be observed with varying number and position of phenolic OH-groups. Caffeoyl-CoA and *p*-coumaroyl-CoA were tested as cinnamoyl moiety and other cinnamoyl-CoA esters are under investigation. The saturation concentration of both caffeoyl- and *p*-coumaroyl-CoA were at 0.2 mM. Saturation for 4-hydroxyphenyllactic acid and 3,4-dihydroxyphenyllactic acid was achieved at 0.5 mM. Up to now it was not possible to decide, which substrates are the natural ones for rosmarinic acid biosynthesis. Possibly the less hydroxylated substrates *p*-coumaroyl-CoA and 4-hydroxyphenyllactic acid are the substrates used in the living cell. This would require one or two hydroxylases to introduce the meta-hydroxyl-groups to the molecule synthesized by rosmarinic acid synthase. These enzymes or an enzyme hydroxylating 4-hydroxyphenyllactic or 4-hydroxyphenylpyruvic acid have to be found in our cell cultures. A similar situation was found in parsley cell cultures. There a 5-O-(4-coumaroyl)shikimate 3'-hydroxylase introduces the OH-group to the coumaroyl moiety of 5-O-(4-coumaroyl)shikimate to form 5-O-caffeoylshikimate [15].

A complete characterization of rosmarinic acid synthase and of the hydroxyphenylpyruvate reductase is in progress.

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