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Purification of rosmarinic acid synthase from cell cultures of *Coleus blumei* Benth.

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Abstract. Rosmarinic acid synthase from cell cultures of *Coleus blumei* Benth. was purified to apparent homogeneity by fractionated ammonium sulfate precipitation (60–80% saturation), hydrophobic interaction chromatography, affinity chromatography and gel filtration. This purification procedure resulted in a 225-fold-enriched specific enzyme activity with a yield of 9%. The protein preparation was apparently pure according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis. The apparent molecular mass determined by gel filtration and SDS-PAGE was 77 kDa, indicating that rosmarinic acid synthase is a monomeric enzyme.

Key words: *Coleus* (cell cultures) – Hydroxycinnamic acid ester – Rosmarinic acid – Rosmarinic acid synthase (purification)

Introduction

The biosynthesis of rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, has been under investigation since 1970 when Ellis and Towers found the precursors for this compound to be the amino acids phenylalanine and tyrosine. Their feeding experiments with radioactively labelled amino acids showed that phenylalanine was only incorporated into the caffeic acid part of RA whereas tyrosine gave rise to the 3,4-dihydroxyphenyllactic acid moiety in plants of *Mentha* (Ellis and Towers 1970) and cell cultures of *Coleus blumei* (Razzaque and Ellis 1977). The enzymes involved in the biosynthesis of RA have successively been identified, isolated and characterized and a scheme for the biosynthetic pathway of RA in cell cultures of *Coleus blumei* has been proposed recently (Petersen et al. 1993). Phenylalanine is converted to 4-coumaroyl-CoA by the enzymes of the general phenylpropanoid pathway, phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase and 4-coumarate CoA-ligase. Tyrosine is transformed to 4-hydroxyphenylpyruvate in a transamination reaction with 2-oxoglutarate catalyzed by tyrosine aminotransferase (De Eknamkul and Ellis 1987a,b; Mizukami and Ellis 1991). 4-Hydroxyphenylpyruvate is further reduced to 4-hydroxyphenyllactate by hydroxyphenylpyruvate reductase (Petersen and Alfermann 1988; Häusler et al. 1991). Rosmarinic acid synthase catalyzes the transfer of the 4-coumaroyl moiety of 4-coumaroyl-CoA to the aliphatic hydroxyl group of 4-hydroxyphenyllactate and hereby forms the first ester, 4-coumaroyl-4′-hydroxyphenyllactate. Since this enzyme activity was first detected with caffeoyl-CoA and 3,4-dihydroxyphenyllactate as substrates, resulting in a direct formation of RA, the enzyme was named rosmarinic acid synthase (RAS) (Petersen and Alfermann 1988; Petersen 1991; Petersen et al. 1993). The first ester 4-coumaroyl-4′-hydroxyphenyllactate is hydroxylated in positions 3 and 3′ of the aromatic rings by membrane-bound hydroxylase activities giving rise to RA (Petersen et al. 1993).

Rosmarinic acid synthase has been fully characterized from partially purified enzyme extracts from suspension-cultured cells of *Coleus blumei* (Petersen and Alfermann 1988; Petersen 1991). In these cell cultures the rate of RA biosynthesis as well as RAS activity can be stimulated by cultivation in medium with 4% sucrose compared with only 2% sucrose in the maintenance medium. However the activity of RAS is enhanced only 2.3-fold in cultures with a 6.5-fold-stimulated RA accumulation (Petersen 1991). This indicates that the high RA content of the cells at the end of the culture period may be the result of an enhanced RAS activity or quantity and a prolonged period of RA synthesis and accumulation. Our ultimate interest is in the mechanism by which RA biosynthesis is regulated on enzyme and gene levels in suspension cultures of *Coleus blumei.* In order to investigate the enzymes and as a step towards the future isolation of the

Abbreviations: DTT = dithiothreitol; HIC = hydrophobic interaction chromatography; RA = rosmarinic acid; RAS = rosmarinic acid synthase; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

respective genes, we attempted to purify the relevant enzymes of RA biosynthesis. We here report on the purification of RAS, the most characteristic and typical enzyme of the RA biosynthetic pathway, to apparent homogeneity.

Materials and methods

Cell cultures. Cell cultures of *Coleus blumei* Benth. were established from a callus culture, which was a gift from Dr. B. Ulbrich (Rhône-Poulenc-Rorer, Köln, FRG), and were cultivated as described previously (Petersen and Alfermann 1988). For the isolation of RAS, suspension cultures which showed an enhanced RA biosynthesis in CB-medium (a modified B5-medium, Gamborg et al. 1968) with 4% sucrose were used.

Chemicals. 4-Coumaroyl-CoA, caffeoyl-CoA and 3,4-dihydroxyphenyllactic acid for the determination of RAS activity were prepared as described previously (Petersen and Alfermann 1988); 4-hydroxyphenyllactic acid was purchased from Sigma (Deisenhofen, FRG).

Buffers. The following buffers were used for extraction and purification of RAS: buffer A: 0.1 M KH_2PO_4/K_2HPO_4, 1 mM dithiothreitol (DTT), pH 7.0; buffer B: 10 mM K_2HPO_4, 1 M $(NH_4)_2SO_4$, 1 mM DTT, pH 7.0 (NH_4OH); buffer C: 10 mM KH_2PO_4/K_2HPO_4, 1 mM DTT, pH 7.0.

Extraction and purification of RAS. Extraction, precipitation and concentration steps were carried out at 0–4°C; chromatographic steps were performed at room temperature with a Waters Advanced Protein Purification System 650 (Waters-Millipore GmbH, Eschborn, FRG).

Suspension cells of *Coleus blumei* were harvested by vacuum filtration after 7 d of growth. The cells were homogenized for 3 × 1 min with an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen i. Br., FRG) together with 1/10 of their fresh weight of Polyclar AT (Serva, Heidelberg, FRG) and 0.67 ml buffer A per gram fresh weight. The homogenate was filtered through a Nylon screen and centrifuged at 48000 · g for 15 min.

The supernatant was used for a fractionated ammonium sulfate precipitation between 60 and 80% saturation using a saturated $(NH_4)_2SO_4$ solution adjusted to pH 7.0 with NH_4OH. The precipitate was collected by centrifugation at 48000 · g for 20 min and resuspended in buffer B to an approximate protein concentration of 6 mg · ml^{-1}.

This protein preparation (approx. 12 mg protein per run) was subjected to hydrophobic interaction chromatography (HIC) on Fractogel TSK Butyl-650 M (Merck, Darmstadt, FRG; column dimensions 10 mm i.d., 90 mm long). The column was pre-equilibrated with buffer B. After application of the protein the column was washed with 10 ml of buffer B, and bound proteins were eluted by a linear gradient of decreasing $(NH_4)_2SO_4$ concentration (1 M to 0 M in 45 ml buffer C) at a flow of 1 ml · min^{-1}. The elution was monitored photometrically at 280 nm; fractions of 2 ml were collected. Fractions containing RAS activity were subjected to ultrafiltration (PM10 membranes, Amicon, Witten, FRG), resulting in exchange of buffer to buffer C and concentration of the protein.

This concentrated protein from HIC (approx. 3 mg per run) was applied to a Fractogel TSK AF-Blue column (10 mm i.d., 90 mm long) pre-equilibrated with buffer C. After washing with 10 ml of buffer C, a linear gradient from 0 to 1 M KCl in 30 ml buffer C followed at a flow of 1 ml · min^{-1}. Fractions (2 ml) with RAS activity were pooled, desalted and concentrated by centrifugal ultrafiltration (Filtron, Karlstein/Main, FRG).

The last purification step was a gel filtration on a Waters Protein Pak Glass 300 SW column (8 mm i.d., 300 mm long). Buffer C was used for equilibration and elution at a flow of 0.25 ml · min^{-1}. The column was calibrated with low-molecular-weight marker proteins (Pharmacia-LKB, Uppsala, Sweden). Approximately 70 µg concentrated protein from the preceeding chromatographic step was applied to the column per run; fractions of 0.5 ml were collected. Rosmarinic acid synthase eluted in fractions corresponding to a molecular weight of about 77 kDa. Fractions containing RAS activity were pooled and concentrated as described before to a final protein concentration of 205 µg · ml^{-1}.

For storage at −18°C 20% glycerol was added to the protein preparations.

Determination of RAS activity. Enzyme assays for the determination of RAS activity during the purification procedure contained 12.5 µl 0.1 M DTT, 5 µl 12.5 mM ascorbic acid, 5 µl 5 mM caffeoyl-CoA, 5 µl 10 mM 3,4-dihydroxyphenyllactic acid and 10 µl or 30 µl of concentrated protein extracts or column eluate fractions, respectively, and were adjusted to a total volume of 125 µl with 0.1 M KH_2PO_4/K_2HPO_4 buffer pH 7.0. After incubation at 30°C for 10 min the reaction was stopped by the addition of 10 µl 6 N HCl. The assays were extracted twice with 500 µl ethyl acetate, the combined organic phases evaporated and the residues redissolved in 150 µl 50% methanol/50% water with 0.01% H_3PO_4. Rosmarinic acid as reaction product was determined by high-performance liquid chromatography according to Petersen (1991).

Determination of protein concentrations. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

Gel electrophoresis. The purification procedure for RAS was monitored by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), essentially according to Laemmli (1970); the separating gel had an acrylamide concentration of 10%. Bands were visualized by Coomassie staining (Serva-Blue G; Serva, Heidelberg, FRG) and silver staining (Oakley et al. 1980). For two-dimensional gel electrophoresis, non-equilibrium pH gel electrophoresis as the first dimension and SDS-PAGE as the second dimension were used. The proteins were transferred to Immobilon membranes by electroblotting in 50 mM boric acid, 50 mM Tris at 30 V overnight, and were visualized on the membrane by staining with Amido Black (Bauw et al. 1990).

Results

The activity of RAS from cell cultures of *Coleus blumei* can be enhanced by cultivation of the cells in medium with 4% sucrose (Petersen and Alfermann 1988). Highest activities can be observed after 6–8 d of cultivation in this medium. Consequently, cells of this age were used for extraction of the enzyme. Rosmarinic acid synthase was purified by a combination of a fractionated ammonium sulfate precipitation and three chromatographic steps which were performed by high-performance protein-purification methods. For this purpose several purification procedures were optimized, e.g. anion exchange chromatography on diethylaminoethyl (DEAE) and MonoQ resins, HIC, chromatofocusing, affinity chromatography on resins with different ligands (coenzyme A, triazine dyes), and gel filtration. The RAS activity could be enriched by all of these methods except chromatofocusing, where no active enzyme was detectable in the column eluates. A combination of purification procedures with good purification factors led to the purification protocol depicted in Table 1. All enzyme activities during the purification procedure were determined with caffeoyl-CoA and 3,4-dihydroxyphenyllactate as substrates.

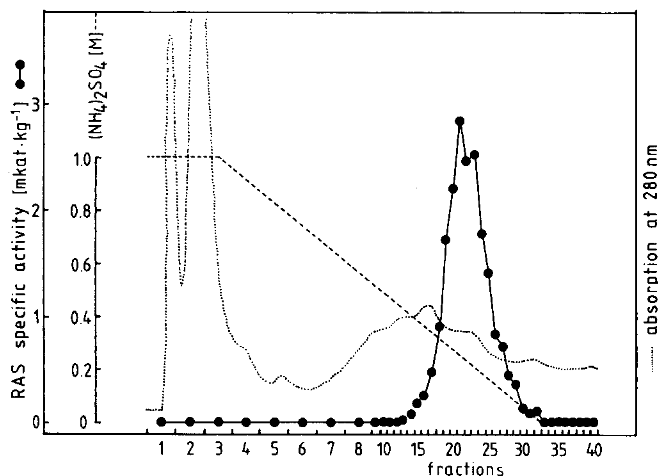

Fig. 1. Elution profile of RAS activity (●--●) from an IC column (Fractogel TSK-Butyl) using a decreasing gradient of $(NH_4)_2SO_4$ (---) in 10 mM potassium phosphate buffer (pH 7) with 1 mM DTT. Fractions of 2 ml were collected at a flow rate of 1 ml · min^{-1}

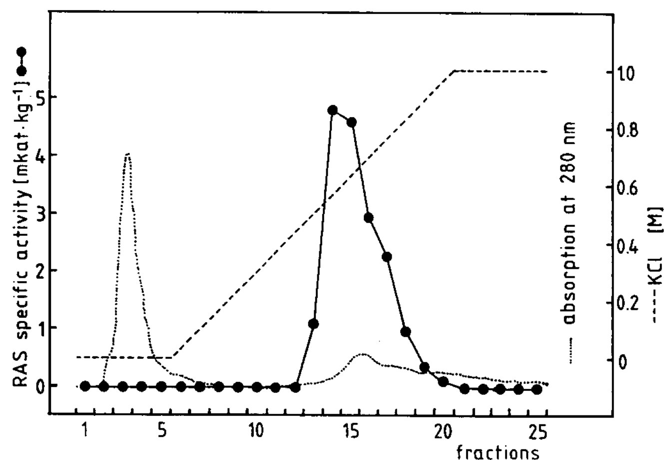

Fig. 2. Affinity chromatography of RAS on a Fractogel TSK AF-Blue column. Elution was performed by a linearly increasing gradient of KCl (---) in 10 mM potassium phosphate buffer (pH 7) with 1 mM DTT at a flow rate of 1 ml · min^{-1}. Fractions of 2 ml were collected

Most of the RAS activity from crude enzyme extracts precipitates between 60 and 80% saturation of ammonium sulfate. This step resulted in a five-fold purification of the enzyme. A considerable part of total RAS also precipitates in the fraction from 50 to 60% saturation of ammonium sulfate. Nevertheless, this fraction was not included in the protein used for purification since the specific activity of RAS was rather low due to contaminating proteins precipitating in this fraction.

The first chromatographic step was HIC (Fig. 1), where RAS was eluted in one peak by a decreasing gradient of ammonium sulfate from 1 M to zero. The shoulder visible in the curve of RAS activity in Fig. 1 was not reproduced in the majority of experiments and does not indicate the presence of RAS isoenzymes. After desalting and concentration, the pooled protein fractions containing RAS activity were loaded onto an affinity chromatography column with Fractogel TSK AF-Blue as matrix. This material is most suitable for nucleotide-requiring enzymes, but enzymes reacting with coenzyme A-esters have also been purified on such matrices with a triazine dye as a ligand (Pharmacia-LKB-booklet Affinity Chromatography; Pharmacia-LKB, Uppsala, Sweden). Rosmarinic acid synthase was bound to this material and was eluted by a linearly increasing gradient of KCl in phosphate buffer, resulting in a single peak with RAS activity (Fig. 2).

As a last purification step after concentrating and desalting the fractions with RAS activity, a gel-filtration step was performed. The column was calibrated with low-molecular-weight proteins. Rosmarinic acid synthase eluted at a position corresponding to a molecular mass of about 77 kDa, again as a single peak (Fig. 3). The overall yield of the purification procedure was 9%, the purification achieved by these four purification steps was 225-fold (Table 1). The relative activity of RAS towards its possible substrates 4-coumaroyl-CoA, caffeoyl-CoA, 4-hydroxyphenyllactate and 3,4-dihydroxyphenyllactate did not change in a way indicating the presence of RAS isoenzymes.

The pooled and concentrated RAS-containing fractions of all steps of the purification procedure were submitted to SDS-PAGE (Fig. 4), essentially according to Laemmli (1970). After the last purification step, only one protein band was visible, migrating at a position corresponding to a molecular mass of about 77 kDa after Coomassie (Fig. 4) and silver staining (not shown).

After two-dimensional gel electrophoresis (1, non-equilibrium pH gel electrophoresis; 2, SDS-PAGE) and protein transfer to Immobilon membranes by electro-

Table 1. Purification protocol for RAS from cell cultures of *Coleus blumei*

Purification step	Protein	Total Protein	Activity	Specific Activity	Purification	Yield
	($\mu g \cdot ml^{-1}$)	(mg)	(pkat)	($\mu kat \cdot kg^{-1}$)	(-fold)	(%)
Crude extract	2390	593.00	29264	49	0	100
$(NH_4)_2SO_4$-precipitation 60–80% sat.	5910	47.30	11576	245	5	40
HIC	1250	3.82	7574	1980	40	26
Affinity chromatogr.	716	0.59	4053	6960	142	14
Gel filtration	205	0.24	2651	11034	225	9

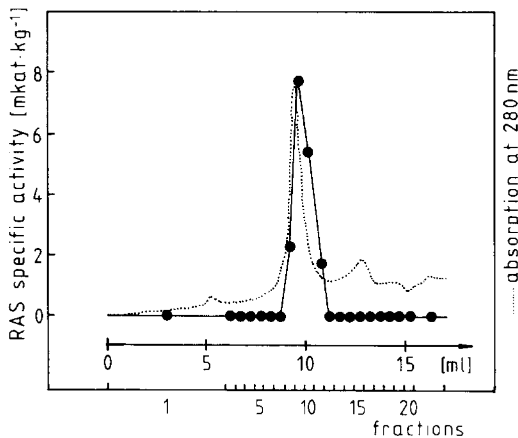

Fig. 3. Gel filtration of RAS on a Waters Protein Pak Glass 300 SW column. The protein was eluted at a flow of 0.25 ml · min^{-1} of 10 mM potassium phosphate buffer (pH 7) with 1 mM DTT. The fraction size was 0.5 ml. The column was calibrated with low-molecular-weight proteins (Pharmacia-LKB)

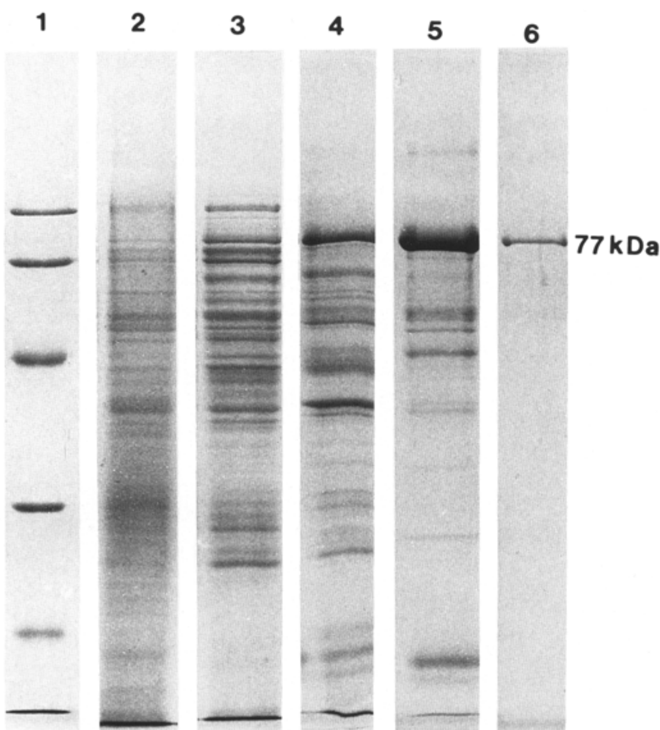

Fig. 4. Discontinuous SDS-PAGE (10%) of RAS at various stages of purification. The gel was stained with Coomassie Blue. *Lane 1*, low-molecular-weight marker proteins (94, 67, 43, 30, 20.1, 14.4 kDa); *lane 2*, crude extract (47 µg protein); *lane 3*, $(NH_4)_2SO_4$-precipitated protein (60–80% saturation; 42 µg protein); *lane 4*, $(NH_4)_2SO_4$ precipitation + hydrophobic interaction chromatography (40 µg protein); *lane 5*, $(NH_4)_2SO_4$ precipitation + HIC + affinity chromatography (24 µg protein); *lane 6*, $(NH_4)_2SO_4$ precipitation + HIC + affinity chromatography + gel filtration (4.9 µg protein)

blotting, only one protein spot corresponding to a molecular mass of 77 kDa was visualized by staining with Amido Black. Thus the protein preparation of RAS appeared to be homogeneous.

The same apparent molecular mass of 77 kDa was determined by gel filtration and SDS-PAGE, indicating that RAS is a monomeric enzyme.

Discussion

This paper deals with the purification of RAS from cell cultures of *Coleus blumei*. Rosmarinic acid synthase catalyzes ester formation during the biosynthesis of RA (Petersen and Alfermann 1988; Petersen 1991). The enzyme transfers the hydroxycinnamoyl-moiety of hydroxycinnamoyl-CoA to the aliphatic hydroxyl group of hydroxyphenyllactate. The putative natural substrates of the enzyme are 4-coumaroyl-CoA and 4-hydroxyphenyllactate giving rise to the first ester 4-coumaroyl-4′-hydroxyphenyllactate (Petersen et al. 1993) although caffeoyl-CoA and 3,4-dihydroxyphenyllactate can be accepted by RAS as well. Rosmarinic acid synthase activity is highest at a stage of the culture period when the suspension cells of *Coleus blumei* have ceased to divide and fresh-weight accumulation is restricted to vacuolization and uptake of water. At this time of the culture period the activity of primary metabolism is decreasing and the protein content of the cells is very low as well. An increase in total protein is only observed until day 2; thereafter the protein content decreases continuously. Therefore, the proportion of enzymes catalyzing RA biosynthesis at this time of the culture period is relatively high compared with the total protein extracted from the cells. This facilitates the purification of RAS. A successful purification was achieved by a combination of a fractionated ammonium sulfate precipitation and three column-chromatographic steps resulting in an apparently homogeneous RAS preparation. Rosmarinic acid synthase behaved like a monomeric enzyme with an apparent molecular mass of 77 kDa.

Tyrosine aminotransferase (TAT) is the only other enzyme involved in RA biosynthesis that has been highly purified up to now (De-Eknamkul and Ellis 1987b). The enzyme was isolated from cell cultures of *Anchusa officinalis*. Here, as described for the purification of RAS from cell cultures of *Coleus blumei*, a rather low purification factor (although related to a loss of activity during storage) resulted in a very pure enzyme preparation. Several isoforms of TAT were detected which showed different substrate specificities. Only one isoenzyme, TAT-1 (and TAT-2 as an artefact of TAT-1) was shown to be a real tyrosine aminotransferase with an activity profile correlated to RA biosynthesis in this cell culture.

The purified RAS protein will allow us to raise antibodies against RAS and to microsequence the protein in order to isolate cDNA clones and genes encoding for this enzyme. This will help us to elucidate the regulation of this enzyme in relation to RA biosynthesis in cell cultures of *Coleus blumei*.

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