Hydroxyphenylpyruvate Reductase from Cell Suspension Cultures of *Coleus blumei* Benth.

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Cell suspension cultures of *Coleus blumei* Benth. producing high amounts of rosmarinic acid were used to study the biosynthetic pathway of this caffeic acid ester. One of the involved enzymes, the hydroxyphenylpyruvate reductase (HPPR), is characterized in this paper. HPPR catalyzes the NAD(P)H dependent reduction of *p*-hydroxyphenylpyruvate to *p*-hydroxyphenyllactate. The enzyme developed maximal activity at an incubation temperature of 37 °C and at a pH of 6.5 to 7.0. The reaction proceeded linearly for an incubation time of 60 min and up to a protein concentration of 0.2 mg per assay. As electron donor HPPR accepted NADH and NADPH with K_m-values of 190 μM and 95 μM respectively. The enzyme reduced differently substituted hydroxyphenylpyruvates but not β-phenylpyruvate. The apparent K_m-values for the various substrates were at 10 μM for *p*-hydroxyphenylpyruvate, at 130 μM for 3,4-dihydroxyphenylpyruvate and at 250 μM for 3-methoxy-4-hydroxyphenylpyruvate. HPPR was competitively inhibited by rosmarinic acid and pyruvate with K_i-values of 210 μM and 200 μM respectively. Caffeic acid, *p*-coumaric acid and cinnamic acid did not affect the enzyme activity but *p*-coumaroyl-CoA inhibited HPPR.

Introduction

Rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (DHPL), is a common secondary compound in species of the Lamiaceae and Boraginaceae. It is of pharmaceutical interest as it has antiinflammatory, antiviral and antibacterial properties. Beside from this the biosynthesis of RA is of considerable interest for the elucidation of regulatory steps in secondary metabolism. The system of RA biosynthesis has proven to be a suitable model, because this pathway consists of only a few enzymatic steps. Furthermore, cell suspension cultures of *Coleus blumei* accumulate almost exclusively RA and in very high amounts (up to 20% of the dry weight of the cells [1]). The RA production of the used suspension of *C. blumei* can be enhanced by raising the sucrose content of the culture medium from 2% to 4% [1]. The enzymes of RA biosynthesis are now known to a great part. Ellis and Towers [2] showed that the caffeic acid moiety of the RA molecule is derived from phenylalanine, whereas the 3,4-dihydroxyphenyllactic acid part is formed from tyrosine. Phenylalanine is metabolized by the enzymes of the general phenylpropanoid metabolism, *i.e.* phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase and cinnamic acid CoA-ligase [3]. The entrypoint enzyme of the tyrosine-derived pathway of RA biosynthesis has been identified to be tyrosine aminotransferase [4]. Petersen and Alfermann [1] have shown that the product of the transamination of tyrosine, *p*-hydroxyphenylpyruvate (pHPP), is reduced to *p*-hydroxyphenyllactate (pHPL) by a specific reductase. The two moieties of the parallel pathways are connected by rosmarinic acid synthase in a transesterification reaction. To date it is not known precisely at which stage of the biosynthesis the two hydroxyl groups in position 3 of the phenolic rings of the RA molecule are inserted.

The purpose of the present paper is the characterization of the hydroxyphenylpyruvate reductase and to report additional information about the regulation of the biosynthesis of RA and the hydroxylation pattern of the substrates of biosynthesis.

Abbreviations: DHPL, 3,4-dihydroxyphenyllactate; DHPP, 3,4-dihydroxyphenylpyruvate; DTT, dithiothreitol; HPPR, hydroxyphenylpyruvate reductase; pHPL, *p*-hydroxyphenyllactate; pHPP, *p*-hydroxyphenylpyruvate; RA, rosmarinic acid.

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Materials and Methods

Cell suspension cultures

Suspension cultures of *Coleus blumei* Benth. were derived from a callus culture provided by Dr. B. Ulbrich (A. Nattermann & Cie., Köln). The suspension cultures were cultivated as described previously [1] and were subcultivated weekly. For the stimulation of rosmarinic acid biosynthesis the cells were transferred into medium with 4% sucrose [1].

Chemicals

3,4-Dihydroxyphenylpyruvate was a gift from Dr. N. Rao, Forschungszentrum Jülich GmbH, F.R.G. 3,4-Dihydroxyphenyllactate was prepared by enzymatic hydrolysis of rosmarinic acid as described previously [1].

Preparation of enzyme extracts

7 days old suspension cultures of *C. blumei* grown in a medium with 4% sucrose were filtered under suction. The following extraction steps were performed at 0–4 °C. The cells were homogenized with an Ultra-Turrax (Janke und Kunkel, Staufen i. Br./F.R.G.) for 90 sec in 0.5 ml buffer (0.1 M KH_2PO_4/K_2HPO_4, 1 mM DTT, pH 7.0) per g fresh weight together with 10% Polyclar AT. The homogenate was passed through a nylon net and the filtrate was centrifuged at 12,000 × *g* for 15 min. The supernatant was fractionated by $(NH_4)_2SO_4$ precipitation (50–60% saturation) by addition of a saturated $(NH_4)_2SO_4$ solution, which was adjusted to pH 7.0 with NH_3. The precipitated protein was sedimented at 48,000 × *g* for 20 min, redissolved in a minimum volume of extraction buffer and desalted on a Sephadex G25-column (Pharmacia, LKB, Uppsala, Sweden).

Assay for HPPR

A standard assay contained in a final volume of 0.25 ml buffer (as above, without adding DTT): 0.25 µmol 3,4-dihydroxyphenylpyruvate (DHPP), 0.5 µmol NADH, 10 nmol ascorbate and 1 µmol DTT. The reaction was started by the addition of 25 µl (approximately 30–40 µg protein) of desalted enzyme preparation. After 10 min incubation at 30 °C it was stopped by adding 25 µl 6 N HCl. For kinetic studies the reaction time was 5 min. The reaction product was extracted three times with 0.5 ml 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 H_3PO_4 (85%). The products were identified and quantified by HPLC using a Shandon Hypersil ODS column (5 µm particle size, 29 cm × 4.6 mm) and isocratic elution with 20% methanol/80% water containing 100 µl/l H_3PO_4 (85%). The flow rate was 1.5 ml min^{-1}. The reaction products were detected spectrophotometrically at 280 nm.

Protein determination

Protein concentrations were determined according to Bradford [5] using BSA as a standard.

Results

HPPR activity during the culture cycle of Coleus blumei suspension cultures

During the characterization of a suspension culture of *Coleus blumei* it was demonstrated that HPPR activity rises in correlation with RA accumulation (Fig. 1). RA productions starts during the growth phase and probably correlates with the depletion of phosphate from the medium. While the RA content of the cells is enhanced from about 1% of the dry weight in medium containing 2% sucrose to over 10% in medium with 4% sucrose, the specific activity of HPPR is increased approximately fourfold. The enzyme assays for the characterization of HPPR are performed with extracts from cells in the high-producing stage during which also HPPR activity is optimal. In cell cultures of *C. blumei* which produce less than 1% of RA on dry weight basis HPPR activity is not detectable.

Enzyme properties

HPPR is a soluble enzyme which can be precipitated between 50 and 60% saturation with ammonium sulphate. It reaches optimal activity at an incubation temperature of 35–37 °C and at a pH of 6.5–7.0. Under standard assay conditions the reaction proceeds linearly for an incubation time of 60 min and up to a protein concentration of 0.2 mg per assay. The examination of different antioxidants or effectors on SH-residues shows that

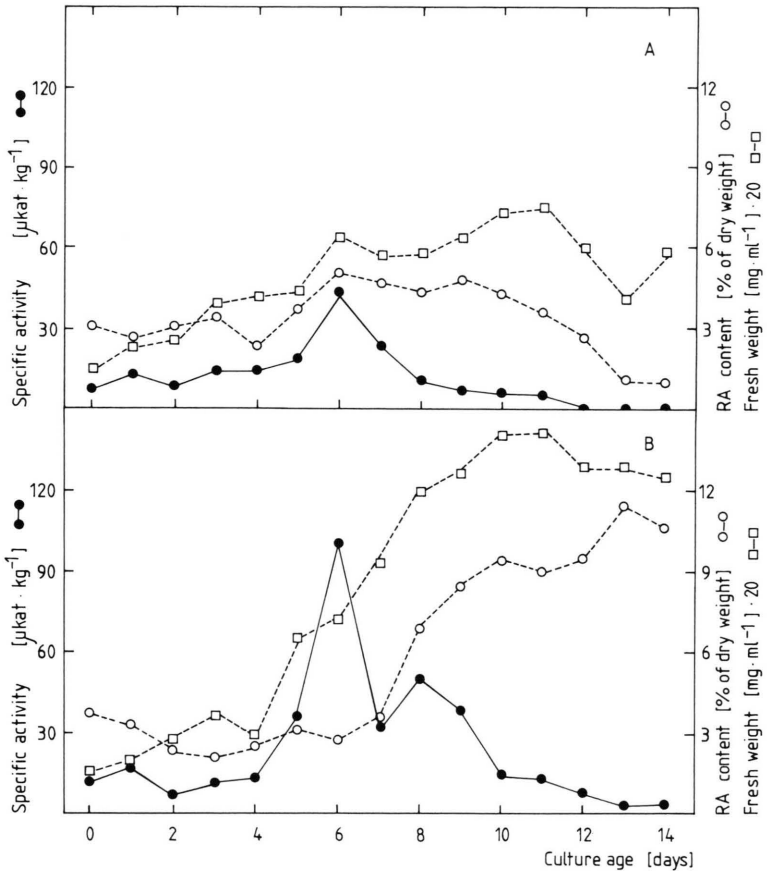

Fig. 1. Cell growth (□), rosmarinic acid accumulation (○) and HPPR activity (●) in suspension cultures of *Coleus blumei* during a culture period of 14 days. A) Parameters in CB_2-medium with 2% sucrose, B) in CB_4-medium with 4% sucrose.

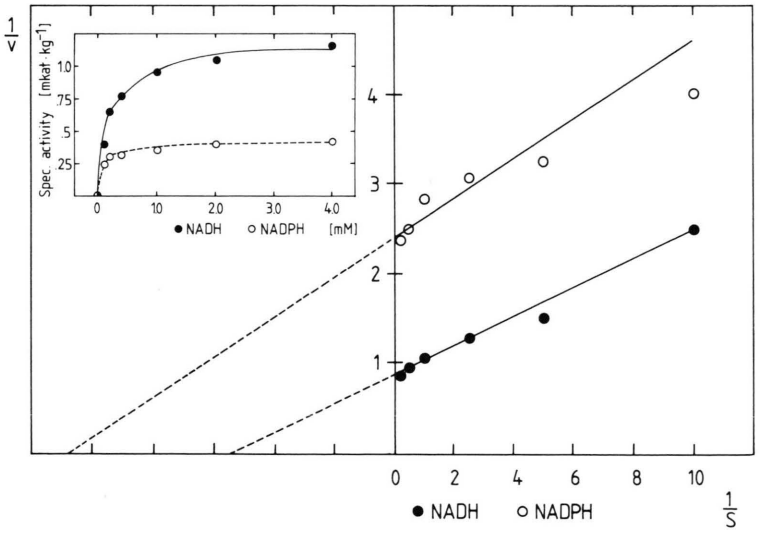

Fig. 2. Determination of the apparent K_m-values of HPPR with NADH (●) and NADPH (○) as substrates. The K_m-values were determined at 190 μM for NADH and at 95 μM for NADPH. The inset shows the kinetic data from which the Lineweaver Burk-plots are derived.

neither ascorbic acid nor DTT nor *p*-hydroxymercurybenzoic acid influence the enzyme activity significantly. To exclude any disturbing effects of phenolases in the enzyme extract, the influence of diethyldithiocarbaminate, an inhibitor of phenolases, on HPPR activity was tested. It appears that this substance does not have any effect on the enzyme activity and thus phenolases also do not influence HPPR.

The direct testing of a reverse reaction, *i.e.* an oxidation of DHPL/pHPL to DHPP/pHPP is not possible as the phenylpyruvates are unstable. However, while studying the influence of the reaction product NAD^+ on HPPR activity, the HPLC diagram shows with rising NAD^+ concentrations an increase of one special peak which can be related to the substrate DHPP. One can therefore assume that the reverse reaction does take place. This observation is supported by the fact that a reduction of NAD^+ is dependent upon the substrates of HPPR and can be measured spectrophotometrically. (An assay contained in a total volume of 0.5 ml 0.1 M KPi-buffer, pH 7.0: 1 μmol NAD^+, pHPL/DHPL in varying concentrations, 10 nmol ascorbate, 1 μmol DTT and 50 μl of desalted enzyme preparation. After 4 min preincubation at 30 °C the reduction of NAD^+ was measured at 366 nm against a reagent blank without pHPL/DHPL. Due to unspecific side reactions it was not possible to establish a spectrophotometrical assay for the quantification of HPPR activity). During storage at −20 °C the enzyme loses 30% of its initial activity within one month, after 10 weeks the loss of activity increases to 85%.

Substrates for HPPR

As electron donor HPPR accepts NADH and NADPH. The K_m-values are at 190 μM for the first and 95 μM for the last (Fig. 2 and Table I). The saturation concentration for both substrates is at 3.0 mM but with NADPH only 30% of the activity with NADH is attained. The enzyme reduces differently substituted hydroxyphenylpyruvates but not β-phenylpyruvate (see Fig. 3 and Table I). HPPR shows the highest affinity for pHPP as a substrate with a K_m of 10 μM. 3,4-Dihydroxyphenylpyruvate and 3-methoxy-4-hydroxyphenylpyruvate (MHPP) also are converted by HPPR with K_m-values of 130 μM and 250 μM respectively. pHPP and DHPP cause a substrate inhibition when they are applied in higher concentrations, whereas MHPP is not inhibitory. Substrate saturation is reached at 100 μM with pHPP, at 500 μM with DHPP and at 3.0 mM with MHPP.

Table I. K_m-values and saturation concentrations of different substrates of HPPR. The K_m-values were determined using Hanes plots and Lineweaver-Burk plots.

Substrate	K_m [μM]	Saturation concentration [mM]
pHPP	10	0.1
DHPP	130	0.5
MHPP	250	3.0
β-PP	–[a]	–
NADH	190	3.0
NADPH	95	3.0

[a] Not accepted as substrate.

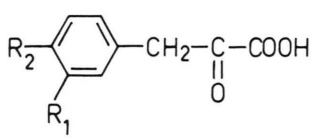

Fig. 3. The structure of the chosen phenylpyruvates which have been investigated as substrates for HPPR. $R_1=R_2=H$, β-phenylpyruvate (β-PP); $R_1=H$, $R_2=OH$, *p*-hydroxyphenylpyruvate (pHPP); $R_1=R_2=OH$, 3,4-dihydroxyphenylpyruvate (DHPP); $R_1=OCH_3$, $R_2=OH$, 3-methoxy-4-hydroxyphenylpyruvate (MHPP).

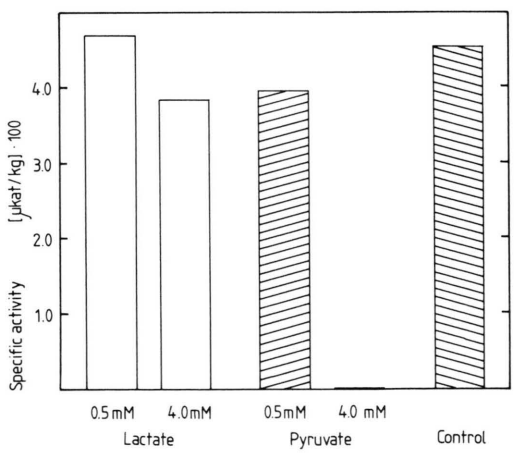

Fig. 4. Effect of lactate and pyruvate on HPPR activity. After preincubating parallel enzyme assays for 10 min with lactate or pyruvate 2 mM DHPP has been added. HPPR is blocked by pyruvate but not by lactate. A standard assay served as control.

Inhibition of HPPR

Various potential intermediates of RA biosynthesis were investigated for their influence on HPPR activity with the aim of detecting a possible mechanism of regulation of the biosynthesis. Caffeic acid and its CoA-ester, *p*-coumaric acid, cinnamic acid, cinnamoyl-CoA and coenzyme A do not affect HPPR activity, *p*-coumaroyl-CoA inhibits the enzyme. At a concentration of 0.5 mM the inhibition reaches 50%. The enzyme is competitively inhibited by RA and pyruvate with K_i-values of 210 µm for the first and 200 µm for the last.

The reduction of pHPP is completely blocked by preincubation with pyruvate (Fig. 4) but lactate has no significant effect.

Stereospecificity of the reaction

It is not possible to isolate an adequate amount of the reaction product to determine its stereochemistry polarimetrically but during incubation of crude enzyme extracts with pHPP, NADH and caffeoyl-CoA rosmarinic acid is formed. This is due to a further metabolism of the reaction product of HPPR by rosmarinic acid synthase. As it is known that rosmarinic acid synthase only accepts the *R*(+)-stereoisomer of the phenyllactate, it is concluded that also the *R*(+)-stereoisomer of pHPL is formed by HPPR.

Discussion

HPPR is an enzyme involved in RA biosynthesis isolated from cell cultures of *Coleus blumei*. The activity of this enzyme is strictly correlated with the synthesis and accumulation of RA. The enzyme can be isolated from the soluble fraction of a cell extract and may be enriched by $(NH_4)_2SO_4$-precipitation.

Experiments concerning the substrate specificity of HPPR show that from the range of tested phenylpyruvates the enzyme has the highest affinity for pHPP with a K_m-value of 10 µM. As electron donor NADPH is accepted by HPPR with a K_m-value of 95 µM, while the K_m-value for NADH is 190 µM. From the K_m to v_{max}-ratios it is concluded that pHPP and NADPH could be the natural substrates *in vivo*. These data corroborate the assumption that hydroxylation at position 3 of the aromatic ring of the DHPL-moiety of the RA molecule takes place at a later stage of RA biosynthesis. Some of the experiments can give information about the structure of HPPR or its reaction centre. The fact that HPPR activity is neither affected by DTT, which protects SH-residues of proteins, nor by *p*-hydroxymercurybenzoate, which destroys them, leads to the conclusion that SH-groups do not play an important role in the active centre of the protein. The OH-group in para-position of the phenylpyruvate seems to be essential for the fixation of the molecule, since the phenylpyruvate without this *p*-hydroxy-function is not accepted by HPPR.

De-Eknamkul and Ellis [6] and Zenk [7] have shown that RA accumulation occurs during the growth phase of *Anchusa officinalis* and *Coleus blumei* cells. This observation is confirmed by the experiments with our cultivar of *C. blumei* (Fig. 1). In all instances RA synthesis begins when phosphate is depleted from the medium. As a specific enzyme of RA biosynthesis HPPR shows a close relation to RA production, as well as the other enzymes involved in RA biosynthesis. The coordinated rises of enzyme activities indicate that the parallel pathways may be coordinated. This assumption is supported by the observation that only small amounts of intermediates of RA biosynthesis can be found in the cells and that an exogenous supply of these intermediates cannot stimulate RA production [8]. It has been reported that PAL can be inhibited by pHPP in *Coleus blumei* cells [9] and tyrosine aminotransferase by DHPL in *Anchusa officinalis* cultures [10]. RA inhibits HPPR, rosmarinic acid synthase [11], cinnamic acid-coenzyme A ligase (unpublished results) and two of three TAT activities in *Anchusa* cells [10]. This again suggests a coordinated regulation of the two pathways. The inhibitory effect of *p*-coumaroyl-CoA on HPPR may indicate a coupling of the parallel pathways. To date a positively acting effector has not been found.

The purification of HPPR and other enzymes of RA biosynthesis as well as studies on the localization and transport of RA will provide further insight into the mechanisms of RA biosynthesis.

Acknowledgements

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