Hydroxyphenylpyruvate Reductase from Cell Suspension Cultures of Coleus blumei Benth.

Elisabeth Häusler, Maike Petersen, and August W. Alfermann

Heinrich-Heine-Universität Düsseldorf, Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Universitätsstraße 1, D-4000 Düsseldorf 1, Bundesrepublik Deutschland

Z. Naturforsch. 46c, 371 – 376 (1991); received January 21 /February 26, 1991

Coleus blumei, Lamiaceae, Cell Suspension Culture, Biosynthesis, Rosmarinic Acid

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-dihydroxyphenyllactic acid; DTT, dithiothreitol; HPPR, hydroxyphenylpyruvate reductase; pHPL, p-hydroxyphenyllactate; pHPP, p-hydroxyphenyllactic acid; RA, rosmarinic acid.

Reprint requests to Elisabeth Häusler.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen 0939–5075/91/0500–0371 $ 01.30/0
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 KH₂PO₄/K₂HPO₄, 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 x g for 15 min. The supernatant was fractionated by (NH₄)₂SO₄ precipitation (50–60% saturation) by addition of a saturated (NH₄)₂SO₄ solution, which was adjusted to pH 7.0 with NH₃. The precipitated protein was sedimented at 48,000 x 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 (DHP), 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 H₃PO₄ (85%). The products were identified and quantified by HPLC using a Shandon Hypersil ODS column (5 μm particle size, 29 cm x 4.6 mm) and isocratic elution with 20% methanol/80% water containing 100 μl/l H₃PO₄ (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 CB2-medium with 2% sucrose, B) in CB4-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-hydroxymercu­
curybenzoic acid influence the enzyme activity sig­
nificantly. To exclude any disturbing effects of
phenolases in the enzyme extract, the influence of
diethylthiocarbaminate, an inhibitor of pheno­
lases, on HPPR activity was tested. It appears that
this substance does not have any effect on the en­
zyme activity and thus phenolases also do not in­
fluence 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 reac­
tion 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 as­
sume that the reverse reaction does take place.
This observation is supported by the fact that a re­
duction of NAD⁺ is dependent upon the substrates
of HPPR and can be measured spectrophotometri­
cally. (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 en­
zyme 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 ini­
tial 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

$\text{R}_1$ \text{CH}_2 \text{C}-\text{COOH} \quad 0$

Fig. 3. The structure of the chosen phenylpyruvates
which have been investigated as substrates for HPPR.
$\text{R}_1=\text{R}_2=\text{H}, \ \beta$-phenylpyruvate ($\beta$-PP); $\text{R}_1=\text{H}, \ \text{R}_2=\text{OH},$
p-$\text{hydroxyphenylpyruvate (pHPPP)}; \ \text{R}_1=\text{R}_2=\text{OH}, \ 3,4-$dih­
hydroxyphenylpyruvate (DHPP); $\text{R}_1=\text{OCH}_3, \ \text{R}_2=\text{OH},$
3-methoxy-4-hydroxyphenylpyruvate (MHPP).

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ [μM]</th>
<th>Saturation concentration [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHPPP</td>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td>DHPP</td>
<td>130</td>
<td>0.5</td>
</tr>
<tr>
<td>MHPP</td>
<td>250</td>
<td>3.0</td>
</tr>
<tr>
<td>$\beta$-PP</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>NADH</td>
<td>190</td>
<td>3.0</td>
</tr>
<tr>
<td>NADPH</td>
<td>95</td>
<td>3.0</td>
</tr>
</tbody>
</table>

$^a$ Not accepted as substrate.

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 dif­
ferently substituted hydroxyphenylpyruvates but
not β-phenylpyruvate (see Fig. 3 and Table I).
HPPR shows the highest affinity for pHPPP as a
substrate with a $K_m$ of 10 μM. 3,4-Dihydroxyphen­
ylpyruvate and 3-methoxy-4-hydroxyphenylpyru­
vate (MHPP) also are converted by HPPR with
$K_m$-values of 130 μM and 250 μM respectively.
pHPPP and DHPP cause a substrate inhibition
when they are applied in higher concentrations,
whereas MHPP is not inhibitory. Substrate satura­
tion is reached at 100 μM with pHPPP, at 500 μM
with DHPP and at 3.0 mM with 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.](image)
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 $\mu M$ for the first and 200 $\mu M$ 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$_4$)$_2$SO$_4$-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 $\mu M$. As electron donor NADPH is accepted by HPPR with a $K_m$-value of 95 $\mu M$, while the $K_m$-value for NADH is 190 $\mu M$. 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

This work was supported by the Deutsche Forschungsgemeinschaft.