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Purification, cloning and functional expression of hydroxyphenylpyruvate reductase involved in rosmarinic acid biosynthesis in cell cultures of *Coleus blumei*

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Abstract

Hydroxyphenylpyruvate reductase (HPPR) is an enzyme involved in the biosynthesis of rosmarinic acid in Lamiaceae reducing hydroxyphenylpyruvates in dependence of NAD(P)H to the corresponding hydroxyphenyllactates. The HPPR protein was purified from suspension cells of *Coleus blumei* accumulating high levels of rosmarinic acid by ammonium sulfate precipitation, anion exchange chromatography, hydroxylapatite chromatography, chromatography on 2′,5′-ADP-Sepharose 4B and SDS-polyacrylamide gel electrophoresis. The protein was tryptically digested and the peptides sequenced. Sequence information was used to isolate a full-length cDNA-clone for HPPR (EMBL accession number AJ507733) by RT-PCR, screening of a *C. blumei* cDNA-library and 5′-RACE-PCR. The open reading frame of the HPPR-cDNA consists of 939 nucleotides encoding a protein of 313 amino acid residues. The sequence showed that HPPR belongs to the family of D-isomer-specific 2-hydroxyacid dehydrogenases. The HPPR-cDNA was heterologously expressed in *Escherichia coli* and the protein was shown to catalyse the NAD(P)H-dependent reduction of 4-hydroxyphenylpyruvate to 4-hydroxyphenyllactate and 3,4-dihydroxyphenylpyruvate to 3,4-dihydroxyphenyllactate.

Abbreviations: DTT, dithiothreitol; G6PDH, glucose-6-phosphate dehydrogenase; HPPR, hydroxyphenylpyruvate reductase; IPTG, isopropyl-β-D-thiogalactoside; SDS, sodium dodecyl sulfate

Introduction

Rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, is a common natural product from species of the Lamiaceae and Boraginaceae (Petersen and Simmonds, 2003). It is present in all plant parts and is supposed to act as a preformed, constitutively accumulated defence compound by virtue of its antimicrobial properties (Szabo *et al*., 1999). Rosmarinic acid has attracted interest because of its adstringent, antibacterial, antiviral and antioxidant properties (Parnham and Kesselring, 1985), for example in preparations against *Herpes simplex* infections (Wölbling and Leonhardt, 1994), as a putative cancer-protective compound in food (Ho *et al*., 1994) or as potential preservative in foods and cosmetics (D'Amelio, 1999). The biosynthesis of rosmarinic acid (see Figure 1) has been under scrutiny since 1970 when Ellis and Towers (1970) elucidated the precursors of rosmarinic acid by feeding experiments with radioactively labelled amino acids.

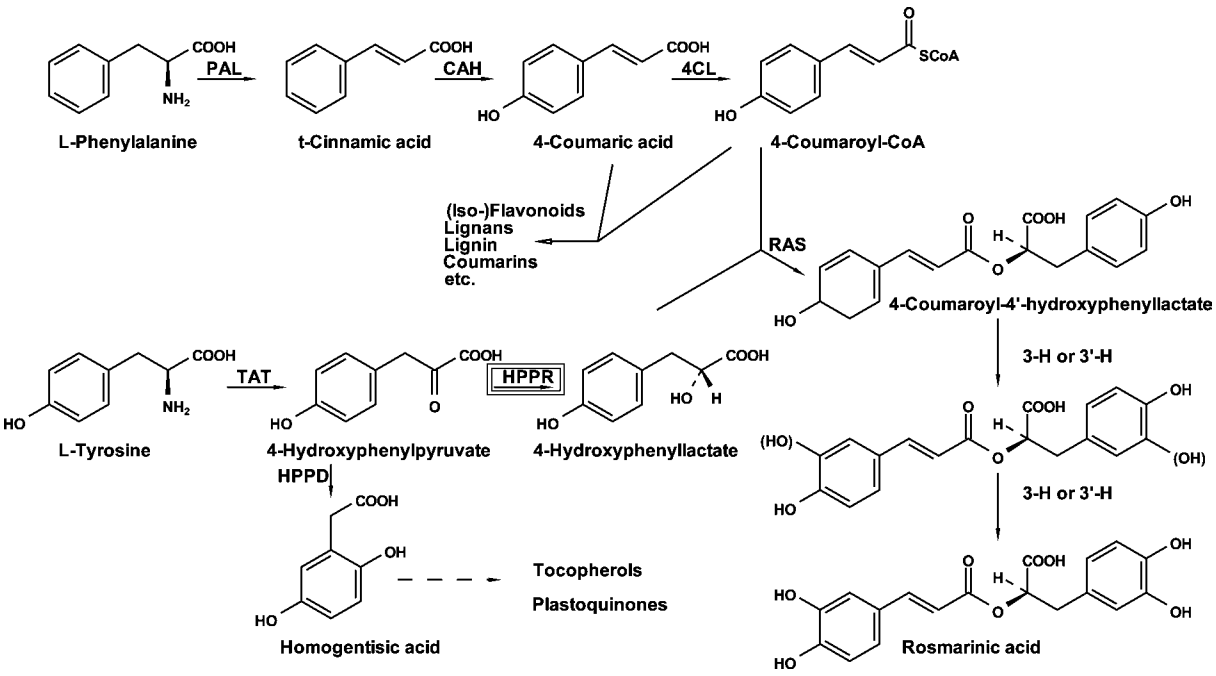

Figure 1. Biosynthesis of rosmarinic acid in *Coleus blumei* and biosynthetic pathways with common precursors. PAL, phenylalanine ammonia-lyase; CAH, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; TAT, tyrosine aminotransferase; HPPR, hydroxyphenylpyruvate reductase (marked by a box); HPPD, hydroxyphenylpyruvate dioxygenase; RAS, 'rosmarinic acid synthase'; 3-H, 3′-H, 3- and 3′-hydroxylases.

L-phenylalanine was found to be the precursor for the caffeic acid moiety, whereas L-tyrosine gave rise to the 3,4-dihydroxyphenyllactic acid part of the molecule. The same result was obtained by Razzaque and Ellis (1977) with rosmarinic acid-accumulating cell cultures of *Coleus blumei*. L-phenylalanine enters the general phenylpropanoid pathway where it is converted to 4-coumaroyl-CoA by phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (CAH) and hydroxycinnamic acid:coenzyme A ligase (4CL) (Petersen *et al.*, 1994, 1995a). L-tyrosine is transaminated by tyrosine aminotransferase (TAT; EC 2.6.1.5) to 4-hydroxyphenylpyruvate (De Eknamkul and Ellis, 1987) which is then reduced by hydroxyphenylpyruvate reductase (HPPR; EC 1.1.1.237) to 4-hydroxyphenyllactate (Petersen and Alfermann, 1988; Häusler *et al.*, 1991; Petersen *et al.*, 1993). HPPR can be considered the first specific enzyme of rosmarinic acid biosynthesis since its substrate 4-hydroxyphenylpyruvate is not only a precursor for rosmarinic acid but also for homogentisic acid which is needed for the formation of tocopherols and probably plastoquinones, both essential in photosynthetically active plant tissues (Hess, 1993; Douce and Joyard, 1996). Since plants are known to form their aromatic amino acids phenylalanine and tyrosine via prephenate and arogenate (Haslam, 1993), 4-hydroxyphenylpyruvate is not an intermediate of aromatic amino acid biosynthesis but has to be formed from L-tyrosine by tyrosine aminotransferase in a transamination reaction (De Eknamkul and Ellis, 1987). 4-Hydroxyphenylpyruvate is then either transformed by hydroxyphenylpyruvate dioxygenase (HPPD; EC 1.13.11.27) to homogentisate or by HPPR to 4-hydroxyphenyllactate which is then coupled with 4-coumaric acid from 4-coumaroyl-CoA to 4-coumaroyl-4′-hydroxyphenyllactate (Petersen and Alfermann, 1988; Petersen, 1991; Petersen *et al.*, 1993). The aromatic 3- and 3′-hydroxyl groups are then introduced by two cytochrome P450-dependent hydroxylation reactions (Petersen, 1997) and rosmarinic acid is formed.

HPPR from suspension cultures of *Coleus blumei* (Lamiaceae) has been fully characterized by Häusler *et al.* (1991). The enzyme accepts 4-hydroxyphenylpyruvate as well as 3,4-dihydroxy-

phenylpyruvate as substrates but needs a free 4-hydroxyl group in the substrate; phenylpyruvate does not serve as substrate. The lowest K_m value was found for 4-hydroxyphenylpyruvate. NADPH as well as NADH are accepted as co-substrates, but the highest affinity was found for NADPH. After first attempts to purify HPPR by Meinhard (1991), we report here on a more advanced purification of this enzyme which eventually led to the isolation of a full-length cDNA clone coding for a protein with HPPR activity from *C. blumei*. According to its sequence, HPPR has to be grouped into the family of D-isomer-specific 2-hydroxyacid dehydrogenases so far mostly known from prokaryotic organisms (Grant, 1989).

Materials and methods

Plant material

Suspension cultures of *Coleus blumei* (Lamiaceae) were grown in CB medium with 2% (CB2) or 4% (CB4) sucrose as reported previously (Petersen and Alfermann, 1988).

Purification and peptide sequencing of HPPR

Suspension cells of *C. blumei* cultivated in CB4 medium for 8 days were harvested by vacuum filtration, frozen in liquid nitrogen and stored at −70 °C. Thawed cells (1000 g) were mixed with 200 g Polyclar 10 and ground in a pre-cooled mortar in a total volume of 1000 ml 0.1 M KH_2PO_4/K_2HPO_4 pH 7.0 containing 1 mM dithiothreitol (DTT). The homogenate was centrifuged at 41 416 × *g* for 20 min at 4–6 °C and the resulting supernatant was used for a fractionated ammonium sulfate precipitation with 40–60% saturation of ammonium sulfate. Precipitated protein was collected by centrifugation at 41 416 × *g* for 20 min at 4–6 °C, redissolved in 50 ml 20 mM bis-Tris-propane/HCl pH 6.0 with 1 mM DTT (buffer A) and desalted on PD-10 columns (Amersham Biosciences). This protein extract was applied to a Q-Sepharose Fast Flow column (Amersham Biosciences; ∅ 5 cm × 12 cm) pre-equilibrated with buffer A. After washing the column with 680 ml buffer A, bound protein was eluted with 1000 ml of a linear NaCl gradient (0–1 M) in buffer A. Fractions of 20 ml were collected, tested for HPPR activity and frozen at −70 °C after addition of glycerol to a final concentration of 13%. Fractions with high HPPR activity were pooled and the protein was precipitated with ammonium sulfate (80% saturation) and collected by centrifugation as described above. The sediment was dissolved in 10 ml 25 mM K_2HPO_4/KH_2PO_4 pH 6.8 containing 1 mM DTT (buffer B) and desalted on PD-10 columns with the same buffer. This protein solution was applied to a hydroxylapatite column (Merck, ∅ 1 cm × 11 cm) pre-equilibrated with buffer B. The column was washed with 20 ml buffer B, then a linearly increasing gradient of 25–250 mM potassium phosphate in 50 ml was used for elution of bound proteins. Fractions of 2 ml were collected, tested for HPPR activity and stored at −20 °C. Pooled fractions from hydroxylapatite chromatography with HPPR activity were applied to a 2′,5′-ADP-Sepharose 4B column (Amersham Biosciences; ∅ 1 cm × 4.5 cm) equilibrated with 10 mM K_2HPO_4/KH_2PO_4 pH 6.5 containing 1 mM DTT and 10% glycerol (buffer C). The column was washed with 16 ml buffer C and then eluted with a linear gradient of 0–1 M NaCl in buffer C in 30 ml. Fractions of 2 ml were collected, tested for HPPR activity and then subjected to electrophoresis on a SDS-polyacrylamide gel (10%) with a 5% stacking gel essentially according to Laemmli (1970). For sample preparation, protein was precipitated with 20% trichloroacetic acid, collected by centrifugation at 16 000 × *g* for 20 min at 4–6 °C, washed twice with ice-cold 80% acetone and dried. The protein was redissolved in 0.1 M Na_2CO_3 containing 10% sucrose, mixed 5:1 with 5× sample buffer (10% SDS, 0.3 M Tris-HCl pH 6.8, 57.5% glycerol, 4% DTT, 0.01% bromophenol blue) and boiled for 5 min in a water bath before loading onto the stacking gel. After electrophoresis for 2–3 h at 300 mA protein bands were visualized by silver staining according to Blum *et al.* (1987). Protein bands representing HPPR were identified by comparing the HPPR activity in single fractions with the staining intensity on the gel. Determination of amino acid sequences of tryptically digested peptides of the putative HPPR band was performed by Dr. Peter Hunziker (Biochemical Institute, Universität Zürich, Switzerland). Prior to sequencing protein bands were stained with Coomassie Brilliant Blue R250 (1.5 g in 100 ml methanol, 5 ml glacial acetic acid, 895 ml water;

destaining solution without Coomassie BB R-250). The protein band in minced gel pieces was subjected to tryptic digestion (0.4 mg trypsin) in 70 μl 0.1 M $(NH_4)HCO_3$, 2 mM $CaCl_2$ pH 7.9 overnight at 37 °C. After removing the gel by centrifugation the supernatant was transferred to 20 μl 5% formic acid, 5% methanol. Of this solution 1 μl was mixed with an equal volume of matrix solution (α-cyano-4-hydroxy cinnamic acid in trifluoroacetic acid/acetonitrile 1:2), dried and used for MALDI-TOF mass spectrometry on a Bruker model Biflex. Aliquots of the tryptic digestion were submitted to electrospray mass spectrometry on a Perkin-Elmer/Sciex model API III after solvent exchange to 60% aqueous methanol/1% formic acid.

Determination of protein concentrations

Protein concentrations were determined according to Bradford (1976) with bovine serum albumin (fraction V) as standard.

Molecular mass determination

The molecular mass of active HPPR was determined by gel filtration on a Superdex 200 HR 10/30 column (Amersham Biosciences; ∅ 1 cm × 30 cm) with 50 mM Na_2HPO_4/NaH_2PO_4 pH 7.0 containing 0.15 M NaCl and 1 mM DTT as eluent at a flow rate of 0.5 ml/min. Low-molecular-weight proteins (12.5–450 kDa) were used for calibration under the same elution conditions.

Isolation of total RNA and mRNA, cDNA synthesis and establishment of the cDNA library

Total RNA was isolated from *C. blumei* suspension cells cultivated for 1–11 days in CB2 and CB4 with the phenol/chloroform method essentially according to Sambrook *et al.* (1989). Poly(A)$^+$ RNA was isolated from 50 μg of the total RNA pool with Dynabeads Oligo $(dT)_{25}$ (Dynal Biotech). cDNA for RT-PCR was prepared from 2 μg poly(A)$^+$ RNA (from 7-day old suspension cells from CB4 medium) with the Omniscript Reverse Transcriptase System (Qiagen) according to the manufacturers' protocol. The cDNA library was established in λgt10 from mRNA isolated from 6-day old suspension cells of *C. blumei* cultivated in CB4 medium as described (Petersen *et al.*, 1995b).

Isolation of a full-length HPPR cDNA clone

Amino acid sequences determined from tryptic peptides (see Figure 3) of the putative HPPR protein were used to design the degenerated DNA primers (HPPR-f, 5′-GC(ACGT)GT(AC-GT)GG (ACGT)AA(CT)(AT)(CG)(ACGT)AA(CT)GC-3′; HPPR-r, 5′-CC(ACGT)A(AG)(ACGT)CC(AGT) AT(AGT)AT(ACG T)CC(ACGT)AC-3′) for RT-PCR. RT-PCR was performed with cDNA from 7-day old suspension cells of *C. blumei* cultivated in CB4 medium (see above) with Hot Star *Taq* polymerase (Qiagen) in assays containing 5 μl 10× buffer, 1 μl 25 mM $MgCl_2$, 1 μl each of 100 μM HPPR-r and HPPR-f, 1 μl 100 mM dNTPs, 2.5 μl cDNA and 0.25 μl Hot Star *Taq* polymerase in a total volume of 50 μl. Best RT-PCR conditions were 39 cycles for 1 min at 95 °C (first cycle 15 min), 2 min at 49 °C (last cycle 5 min), 2 min at 72 °C (last cycle 10 min). The resulting amplicon was ligated into pGEM-T (Promega) and transformed into *Escherichia coli* DH5α and sequenced. The 317 bp fragment was cut from the isolated plasmid with *Cfr*42I and *Nco*I and labelled with the DIG-High-Prime Kit (Roche Diagnostics) for screening of about 60 000 plaque-forming units of the *C. blumei* cDNA library in λgt10 according to the standard protocol of the DIG labelling and detection kit (Roche Diagnostics).

Phage-DNA from positive plaques was purified with Qiagen-tips 20 (Qiagen) according to the manufacturers' protocol, cut with *Eco*RI, ligated into pGEM-4Z (Promega) and transformed into competent *E. coli* DH5α. Sequencing revealed an insert of 865 bp. 5′-RACE-PCR with the Smart-RACE-PCR system (BD Clontech) was used to acquire the 5′ terminus of the HPPR cDNA. Template mRNA was purified from 5- to 7-day old suspension cells of *C. blumei* grown in CB4 medium (see above). The gene-specific primer used for 5′-RACE-PCR was 5′-GCCAAAATCAACCC-GATCGCCAAATCC-3′. PCR conditions were 45 cycles at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min. The 5′-RACE-PCR amplicon was ligated into pGEM-T and transformed into *E. coli* DH5α. Plasmid DNA was isolated from several individual transformants and sequenced.

The full-length open reading frame of the putative HPPR cDNA was amplified by RT-PCR with the forward primer 5′-GGAATTCCAT*ATG*GAGGCGATTGGCGTTTTGATG-3′ and the reverse primer 5′-CAGGATCCTA*TCA* AACTACTGGAGTTAAGAGAG-3′ to introduce 5′-*Nde*I and 3′-*Bam*HI restriction sites containing start and stop codons (italics), respectively, at the sequence termini. Reverse transcription was performed with the RevertAid Kit (Promega) with total RNA from 5- to 7-day old cells of *C. blumei* as template. PCR was performed with the Expand High Fidelity PCR System (Roche Diagnostics) under the following conditions: 38 cycles at 95 °C for 1 min, 60 °C for 2 min and 72 °C for 2 min and, finally, 1 cycle at 95 °C for 1 min, 60 °C for 5 min and 72 °C for 10 min.

Heterologous expression of the putative HPPR cDNA in Escherichia coli

The full-length PCR amplicon was digested with *Nde*I and *Bam*HI (MBI Fermentas) and ligated in-frame into the expression vector pET15b (Novagen) digested with the same restriction enzymes. The plasmid was introduced into *E. coli* BL21(DE3)pLysS made competent with the help of the RotiTransform system (Roth). *E. coli* harbouring pET15b without an insert or pET15b with HPPR cDNA were inoculated into 50 ml Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin. The cells were grown at 37 °C overnight under shaking (200 rpm) and diluted 1:10 with fresh LB medium containing 100 μg/ml ampicillin. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM when bacterial growth was equivalent to a $A_{600} = 1.4$ at 37 °C. After incubation at 37 °C under shaking (200 rpm) for 3 h, bacteria were harvested by centrifugation ($5000 \times g$, 5 min). The bacterial sediments were washed with 30 ml 20 mM 0.1 M Tris–HCl pH 8.0 and stored at –70 °C overnight. Soluble protein was extracted by sonication (0.3 cycles, 100%, 1 min) of the bacteria in 2.5 ml binding buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole) per gram bacterial sediment. The crude extract was centrifuged at $5000 \times g$ for 10 min at 4 °C to yield a cell-free supernatant that was assayed for HPPR activity.

Metal chelate chromatography was performed with Ni-NTA His-Bind Superflow Resin (Novagen) essentially according to the manufacturers' protocol.

Determination of HPPR activity

The assay contained in a final volume of 0.25 ml buffer (0.1 M KH_2PO_4/K_2HPO_4 pH 7.0), 10 nmol ascorbate, 1 μmol DTT, 0.5 μmol NADPH, 0.25 μmol 4-hydroxyphenylpyruvate (Sigma) and 100 μl crude protein extract. After 15 min incubation at 30 °C the reaction was stopped by adding 25 μl 6 M HCl. The assay was extracted three times with 0.5 ml ethyl acetate and the organic solvent was evaporated. The residue was re-dissolved in 100 μl HPLC eluent. The reaction product was identified and quantified by HPLC on a Hypersil ODS column (length 250 mm + pre-column 20 mm, diameter 4 mm) with isocratic elution and detection at 280 nm. The eluent was 10% methanol, 90% water and 0.01% H_3PO_4 (85%). The reaction product 4-hydroxyphenyllactate was identified and quantified with an authentic standard (Sigma).

DNA sequencing

Automated sequencing was performed by Prof. U. Maier and Dr. S. Zauner at the Philipps-Universität Marburg (Fachbereich Biologie, Zellbiologie und Angewandte Botanik) on a LI-COR 4200 sequencer (MWG-Biotech). Cloned DNA was sequenced with IRD700/IRD800-labelled M13-20 standard primers and the Thermosequenase Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Biosciences).

Results

Purification and sequencing of HPPR protein

HPPR was first described as an enzyme of rosmarinic acid biosynthesis from suspension cultures of *C. blumei* (Petersen and Alfermann, 1988) and characterized with respect to its kinetic properties by Häusler *et al.* (1991) in partially purified protein preparations. In order to be able to clone the respective cDNA and gene, first attempts were made to purify this reductase (Meinhard, 1991). A sufficient partial purification of the HPPR protein was finally achieved by a series of ammonium

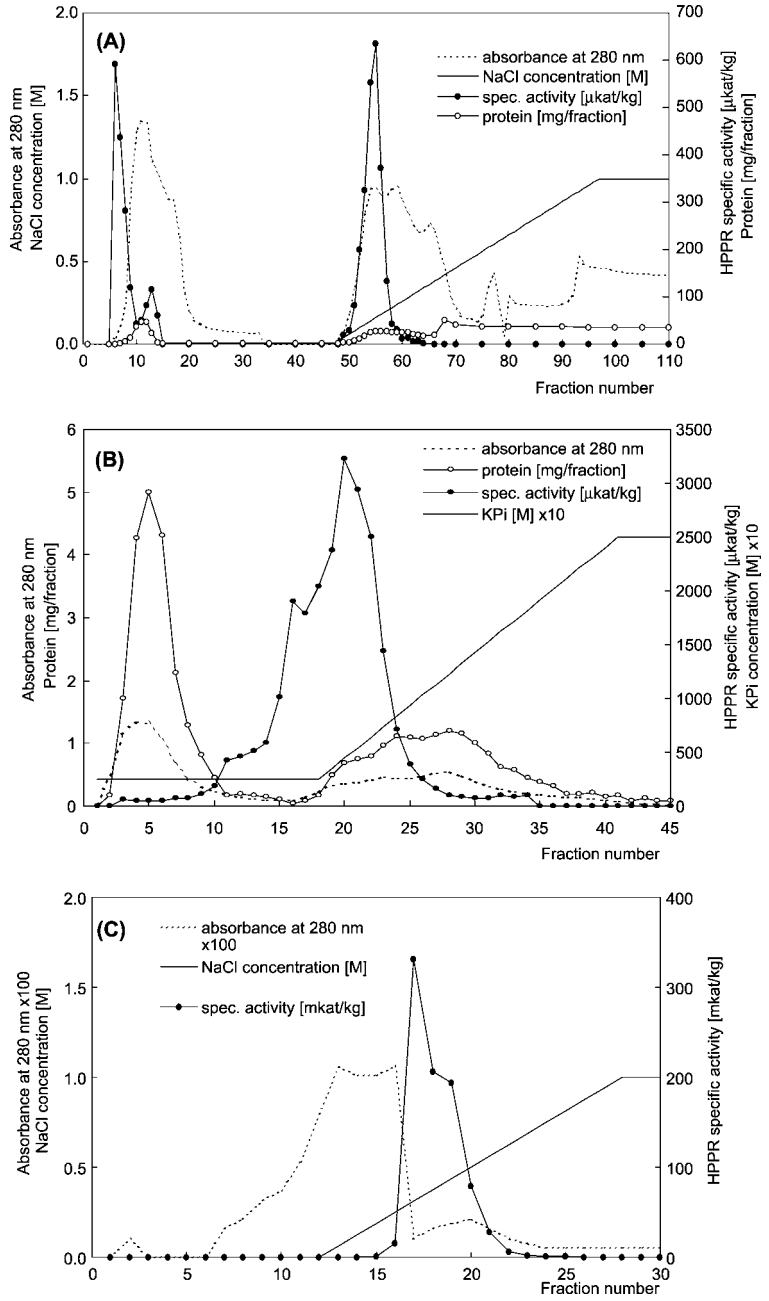

Figure 2. Purification of HPPR from suspension cells of *Coleus blumei*. (A) Anion exchange chromatography on Q-Sepharose Fast Flow, elution with a linear NaCl-gradient from 0 to 1 M. (B) Chromatography on hydroxylapatite, elution with a potassium phosphate gradient from 25 to 250 mM. (C) Affinity chromatography on 2′,5′-ADP-Sepharose 4B, elution with a linear gradient of 0–1 M NaCl.

sulfate precipitation between 40% and 60% saturation of ammonium sulfate and column chromatography on Q-Sepharose Fast Flow, hydroxylapatite and 2′,5′-ADP-Sepharose 4B (Figure 2A–C). This procedure resulted in a 4012-fold purification of HPPR with a yield of 18.6% (Table 1). The molecular mass of active HPPR was determined by gel filtration and resulted in an apparent molecular mass of 45–68 kDa. The fractions of the last purification

Table 1. Purification protocol for HPPR from 1 kg suspension cells of *Coleus blumei* cultivated for 8 days in CB4 medium.

	HPPR activity (pkat)	Total protein (mg)	Specific HPPR activity (μkat/kg)	Yield (%)	Purification factor
Crude cell extract	52 536	2807.00	18.7	100	1.0
$(NH_4)_2SO_4$ precipitation (40–60% saturation)	52 583	654.00	80.5	100	4.3
Q-Sepharose FF	54 096	138.00	391.4	103	20.9
Hydroxylapatite	21 795	10.93	1994.1	41	106.5
2′,5′-ADP-Sepharose 4B	9 763	0.13	75102.4	19	4011.9

step on 2′,5′-ADP-Sepharose 4B were separately tested for HPPR activity, electropherographed on a SDS-polyacrylamide gel and visualized by Coomassie Brilliant Blue and silver staining. The staining intensity of the separated protein bands was compared to HPPR activity. This resulted in the identification of two protein bands with good correlation of staining and activity. A band corresponding to 58.6–62.2 kDa could be identified as glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) by MALDI-TOF analysis of tryptic peptides because of their similarity to the peptide spectrum of G6PDH from *Solanum tuberosum* (SwissProt P37830; Graeve *et al*., 1994). Sequence determination by electrospray mass spectrometry of three peptides supported this identification (peptide 1, YVSGSYDAPDGFQALDK; peptide 2, YQGVVIPEAYER; peptide 3, GPAEADQLLEK). Determination of the activity of G6PDH in the fractions from chromatography on 2′,5′-ADP-Sepharose 4B showed that HPPR and G6PDH coeluted during the purification process (HPPR, fractions 16–23; G6PDH, fractions 16–24; highest specific activity in both cases in fraction 20). The subunits of G6PDH from *S. tuberosum* consist of 511 amino acid residues and have a molecular mass of 58 470 Da. The active enzyme has been described to be a homotetramer (Graeve *et al*., 1994).

Two out of four tryptic peptides of the second protein band corresponding to a molecular mass of 32.6–37.2 kDa showed similarity to a putative D-isomer specific 2-hydroxyacid dehydrogenase from *Arabidopsis thaliana* (SwissProt Q9CA90). This general activity corresponds to the activity of HPPR which oxidizes the D-isomer of the 2-hydroxyacid 4-hydroxyphenyllactate to the 2-oxoacid 4-hydroxyphenylpyruvate or, in rosmarinic acid biosynthesis, catalyses the reverse reaction (4-hydroxyphenylpyruvate → 4-hydroxyphenyllactate). The open reading frame of the nucleotide sequence from *A. thaliana* encodes a protein of 313 amino acid residues with a molecular mass of 34 161 Da and was found by sequencing chromosome 1 of this plant (Theologis *et al*., 2000). The gene from *Arabidopsis* was not expressed heterologously and the activity was never established.

Isolation of a full-length cDNA of the putative HPPR

Sequences of two tryptic peptides (AVVGNSNA and VG(L/I)(L/I)G(L/I)G) of the partially purified putative HPPR protein were used to design degenerated DNA primers for RT-PCR. The degeneration degree was quite high since the amino acids leucine (L) and isoleucine (I) could not be distinguished during sequencing and due to the multiplicity of codons for some of the amino acids in the identified peptides. cDNA for RT-PCR was synthesized from total RNA isolated from 7-day old suspension cells of *C. blumei* grown in CB4 medium. PCR products with an approximate length of 300 nucleotides were observed at all annealing temperatures between 45 and 55 °C, but the strongest signal was seen at 49 °C. After cloning into pGEM-T, the nucleotide sequence of this 317 bp amplicon was determined. The sequence showed 80% identity to the sequence of the putative D-isomer specific 2-hydroxy-acid dehydrogenase from *Arabidopsis* (SwissProt Q9CA90). The 317 bp fragment was labelled with DIG and used to screen a cDNA library from *C. blumei* in λgt10 (Petersen *et al*., 1995b). This resulted in the isolation of a cDNA of 865 nucleotides coding for a protein of 260 amino acid residues and representing the 3′ end of the putative HPPR cDNA. The identity with the putative D-isomer-specific 2-hydroxyacid dehydrogenase from *Arabidopsis*

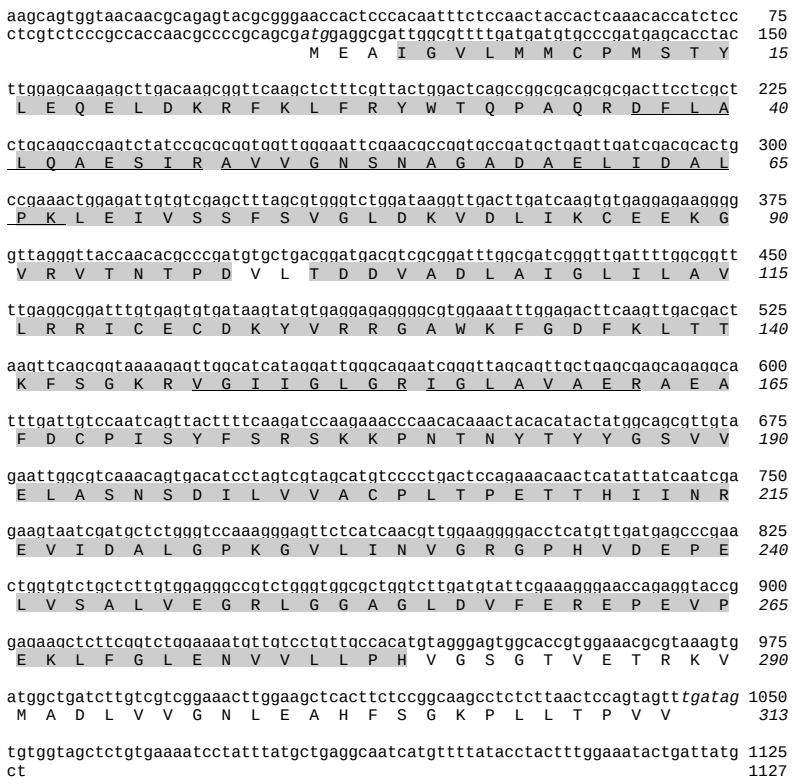

Figure 3. Full-length cDNA sequence for the putative HPPR from *Coleus blumei*. Start and stop codons are printed in italics, the peptides identified by sequencing of the purified HPPR protein are underlined. The catalytic domain (PFAM PF00389, amino acid residues 4–98) and the NAD(P)H-binding domain (PFAM PF02826, amino acid residues 100–279) typical of the family of D-isomer-specific 2-hydroxyacid dehydrogenases are marked in grey.

was 78%. Isolation of the 5′ end of the putative HPPR cDNA was achieved by 5′-RACE-PCR resulting in an amplicon of 400–500 bp. The complete sequence obtained for the putative HPPR (EMBL accession number AJ507733) consisted of 1127 bp with an open reading frame (ORF 106–1044) of 939 nucleotides encoding a protein of 313 amino acid residues (Figure 3) with a calculated molecular mass of 34 113 Da. All four tryptic peptides of the partially purified HPPR protein identified by peptide sequencing could be retrieved in the translated amino acid sequence of the putative HPPR cDNA (Figure 3).

Functional expression of the putative HPPR cDNA in Escherichia coli

The complete open reading frame of the putative HPPR cDNA was amplified by long-distance PCR incorporating at the same time restriction sites for *Nde*I and *Bam*HI. After cleaving the PCR product with these restriction endonucleases the fragment was ligated in-frame into the expression vector pET15b digested with the same restriction enzymes and introduced into *E. coli* BL21(DE3)-pLysS.

For expression studies, the bacteria were grown in liquid LB medium and induced by the addition of 1 mM IPTG. The bacteria were harvested 3 h after induction. The bacterial protein extracts from induced bacteria harbouring the expression plasmid with HPPR cDNA were shown to catalyse the NAD(P)H-dependent reduction of 4-hydroxyphenylpyruvate to 4-hydroxyphenyllactate (Figure 4). This is the same reaction as observed for HPPR from *C. blumei* (Petersen and Alfermann, 1988; Häusler *et al*., 1991). Bacterial protein extracts from bacteria with pET15b without HPPR cDNA cultivated and treated in the same way did not show significant HPPR activity (Figure 4). The low reduction of 4-hydroxyphenylpyruvate in crude extracts from bacteria without

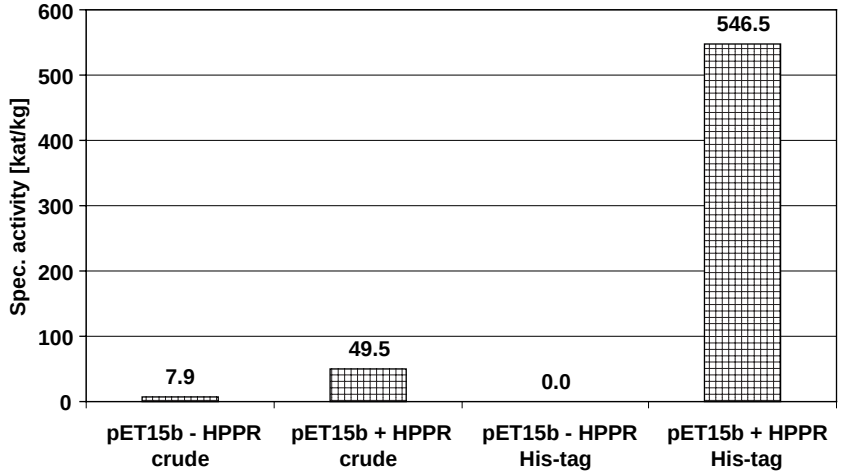

Figure 4. Heterologous expression of HPPR in *E. coli* BL21(DE3)pLysS after induction for 3 h with 1 mM IPTG. pET15b − HPPR, plasmid without open reading frame of HPPR; pET15b + HPPR, plasmid with open reading frame of HPPR; crude, crude bacterial extracts; His-tag, purified by metal chelate chromatography.

HPPR cDNA might be due to lactate dehydrogenase activities from primary metabolism of *E. coli* (Garvie, 1980). This activity was completely abolished after purification of HPPR via its His-tag by metal chelate chromatography (Figure 4). As HPPR from suspension cells of *C. blumei*, the heterologously expressed enzyme accepted as substrate 3,4-dihydroxyphenylpyruvate with the highest specific activity (467/1207 μkat/kg with NADH/NADPH = 100%) followed by 4-hydroxyphenylpyruvate (37%/57%). Reduction of phenylpyruvate occurred only to a very low extent (1.6%/1.8%).

Discussion

Hydroxyphenylpyruvate reductase (HPPR) catalyses the first specific biosynthetic step in the biosynthesis of rosmarinic acid from the aromatic amino acids phenylalanine and tyrosine. 4-Hydroxyphenylpyruvate, formed by transamination of tyrosine, is either a precursor for homogentisic acid, an intermediate of tocopherol biosynthesis, or of 4-hydroxyphenyllactate, a precursor of rosmarinic acid (Figure 1). HPPR was purified by a series of chromatographic steps (Figure 2) and SDS–PAGE and used for sequencing of tryptic peptides by MALDI-TOF and electrospray mass spectrometry. The peptides showed homology to a hitherto unidentified putative D-isomer-specific 2-hydroxyacid dehydrogenase from *Arabidopsis thaliana* (Theologis *et al*., 2000) from a genome sequencing project. This corresponds to the activity of HPPR. A full-length cDNA was isolated on the basis of this peptide information by PCR techniques and screening of a *Coleus blumei* cDNA library. The full-length cDNA clone (Figure 3) could be actively expressed in *Escherichia coli* as a His-tag fusion protein. Bacterial extracts prepared from bacteria carrying the pET15b-plasmid with the HPPR cDNA after IPTG induction showed the expected HPPR enzyme activity (Figure 4). A low activity found in crude extracts from bacteria without the HPPR insert was probably due to lactate dehydrogenase activities commonly found in bacteria (Garvie, 1980). This activity was completely abolished after His-tag purification of the bacterial extracts (Figure 4). Three different aromatic phenylpyruvate derivates were tested for their acceptance as substrate. As the native enzyme from *C. blumei* (Häusler *et al*., 1991), the heterologously expressed HPPR accepted 3,4-dihydroxyphenylpyruvate best, followed by 4-hydroxyphenylpyruvate with about 50% activity. With the native enzyme no activity could be shown with phenylpyruvate and this activity remained very low (about 1.7%) with the heterologously expressed enzyme. This small difference could probably be due to the better analytical methods used now. In contrast to native HPPR (Häusler *et al*., 1991), the enzyme isolated from

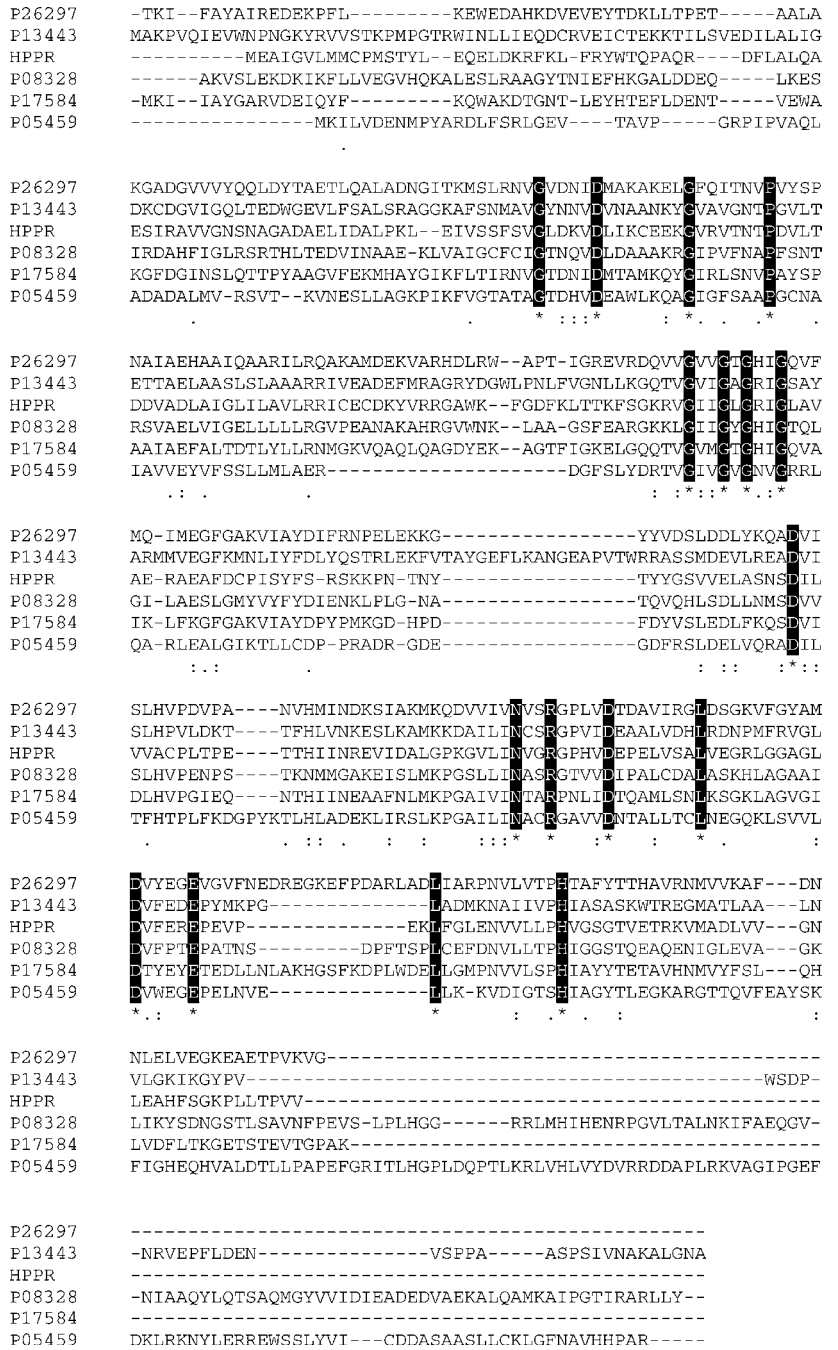

Figure 5. Sequence alignment of amino acid sequences of D-isomer-specific 2-hydroxyacid reductases and HPPR. The 17 conserved amino acid residues are shown in black boxes. P26297, D-lactate dehydrogenase from *Lactobacillus delbrueckii* ssp. *bulgaricus*; P13443, hydroxypyruvate reductase from *Cucumis sativus*; HPPR, hydroxyphenylpyruvate reductase from *Coleus blumei*; P08328, D-3-phosphoglycerate dehydrogenase from *Escherichia coli*; P17584, D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei*; P05459, erythronate 4-phosphate dehydrogenase from *E. coli*.

HPPR-transformed *E. coli* showed higher specific activities with NADPH compared to NADH. This cannot be explained at the moment, but highly purified HPPR from *C. blumei* suspension cells already showed nearly equal activities with the two different electron donors (Meinhard, 1991).

Comparison of the translated amino acid sequences of the putative HPPR and putative D-isomer-specific 2-hydroxyacid dehydrogenases with unknown function from *Arabidopsis thaliana* (Theologis *et al*., 2000) and *Oryza sativa* (EMBL accession number Q8W0A4) showed identities of 76.4% and 68.7%, respectively. The identity to D-2-hydroxyacid dehydrogenases from prokaryotes usually was considerably lower; here highest identities were observed for putative 2-hydroxyacid dehydrogenases from *Agrobacterium tumefaciens* (Wood *et al*., 2001) and *Sinorhizobium meliloti* (Capela *et al*., 2001) with 52% and 44–50%, respectively. A putative NAD(P)H-binding domain was identified in the amino acid residues 100–279 (PFAM PF02826) and a putative catalytic domain in the amino acid residues 4–98 (PFAM PF00389) (see Figure 3). These domains were identified as typical domains for the family of D-isomer-specific 2-hydroxyacid dehydrogenases (InterPro accession nyumber IPR002162; Grant, 1989; Kochhar *et al*., 1992). The NAD(P)H binding motif –G–X–G–X–X–G– is seen in the HPRR sequence at amino acid residues 151–156 and reads –G–L–G–R–I–G– (Figure 3). D-isomer-specific 2-hydroxyacid dehydrogenases have so far mostly been described from prokaryotic organisms. However, hydroxypyruvate reductase (EC 1.1.1.81), being the same enzyme as D-glycerate dehydrogenase (EC 1.1.1.29), is an enzyme of photorespiration and was first cloned from cucumber (Greenler *et al*., 1989; Schwartz *et al*., 1991). D-3-Phosphoglycerate dehydrogenase (EC 1.1.1.95) was found in prokaryotes as well as in eukaryotes as an enzyme of L-serine biosynthesis. The plant enzyme is known from for example *Glycine max* (Boland and Schubert, 1983), *Pisum sativum* (Slaughter and Davies, 1975) and *Triticum aestivum* (Rosenblum and Sallach, 1975). An alignment of prokaryotic and eukaryotic D-isomer-specific 2-hydroxyacid dehydrogenases, namely D-lactate dehydrogenase from *Lactobacillus delbrueckii* ssp. *bulgaricus* (SwissProt accession number P26297), hydroxypyruvate reductase from *Cucumis sativus* (SwissProt P13443), D-3-phosphoglycerate dehydrogenase from *Escherichia coli* (SwissProt P08328), D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei* (SwissProt P17584) and erythronate 4-phosphate dehydrogenase from *E. coli* (SwissProt P05459) with the deduced amino acid sequence of HPPR (Figure 5) revealed 17 conserved amino acid residues. Kochhar *et al*. (1992) also described that 19 amino acid residues were conserved in his alignment of D-isomer-specific 2-hydroxyacid dehydrogenases of which only two are missing in the alignment presented here. Among the conserved amino acid residues is the NAD(P)H-binding motif G–X–X–G–X–G–X–X–G. Of the 17 conserved amino acid residues 13 are found in the NAD(P)H-binding domain (residues 100–279). The lower extent of conservation in the catalytic domain (residues 4–98) correlates with the higher variability of the bound substrates, the different 2-hydroxy acids. However, the two domain structure seems to conserved as well and therefore the three-dimensional structure of HPPR might be similar to the structure of already crystallized prokaryotic dehydrogenases, such as D-glycerate dehydrogenase from *Hyphomicrobium methylovorum* (1gdh; Goldberg *et al*., 1994), phosphoglycerate dehydrogenase from *Escherichia coli* (1psd; Schuller *et al*., 1995), hydroxyisocaproate dehydrogenase from *Lactobacillus casei* (1dxy; Dengler *et al*., 1997) or D-lactate dehydrogenase from *Lactobacillus helveticus* (2dld; Bernard *et al*., 1995).

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