Subcellular localization and purification of a *p*-hydroxyphenylpyruvate dioxygenase from cultured carrot cells and characterization of the corresponding cDNA

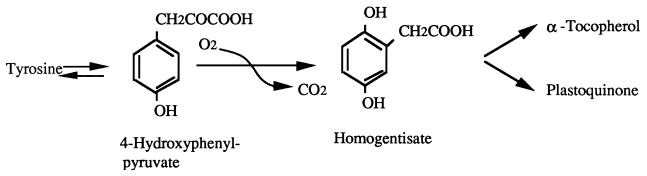
Isabelle GARCIA*, Matthew RODGERS†, Catherine LENNE*, Anne ROLLAND†, Alain SAILLAND† and Michel MATRINGE*[1]

*Unité Mixte CNRS/Rhône-Poulenc (UMR 41), Rhône-Poulenc Agrochimie, 14–20 rue Pierre Baizet, 69263 Lyon Cedex 09, France, and †Département des Biotechnologies, Rhône-Poulenc Agrochimie, 14–20 rue Pierre Baizet, 69263 Lyon Cedex 09, France

p-Hydroxyphenylpyruvate dioxygenase catalyses the transformation of *p*-hydroxyphenylpyruvate into homogentisate. In plants this enzyme has a crucial role because homogentisate is the aromatic precursor of all prenylquinones. Furthermore this enzyme was recently identified as the molecular target for new families of potent herbicides. In this study we examine precisely the localization of *p*-hydroxyphenylpyruvate dioxygenase activity within carrot cells. Our results provide evidence that, in cultured carrot cells, *p*-hydroxyphenylpyruvate dioxygenase is associated with the cytosol. Purification and SDS/PAGE analysis of this enzyme revealed that its activity is associated with a polypeptide of 45–46 kDa. This protein specifically cross-reacts with an antiserum raised against the *p*-hydroxyphenylpyruvate dioxygenase of *Pseudomonas fluorescens*. Gel-filtration chromatography indicates that the enzyme behaves as a homodimer. We also report the isolation and nucleotide sequence of a cDNA encoding a carrot *p*-hydroxyphenylpyruvate dioxygenase. The nucleotide sequence (1684 bp) encodes a protein of 442 amino acid residues with a molecular mass of 48094 Da and shows specific C-terminal regions of similarity with other *p*-hydroxyphenylpyruvate dioxygenases. This cDNA encodes a functional *p*-hydroxyphenylpyruvate dioxygenase, as evidenced by expression studies with transformed *Escherichia coli* cells. Comparison of the N-terminal sequence of the 45–46 kDa polypeptide purified from carrot cells with the deduced peptide sequence of the cDNA confirms that this polypeptide supports *p*-hydroxyphenylpyruvate dioxygenase activity. Immunodetection studies of the native enzyme in carrot cellular extracts reveal that N-terminal proteolysis occurs during the process of purification. This proteolysis explains the difference in molecular masses between the purified protein and the deduced polypeptide.

INTRODUCTION

p-Hydroxyphenylpyruvate dioxygenase (HPPD; *p*-hydroxyphenylpyruvate:oxygen oxidoreductase, hydroxylating, decarboxylating, EC 1.13.11.27, EC 1.14.2.2) catalyses the formation of homogentisate (2,5-dihydroxyphenylacetate) from *p*-hydroxyphenylpyruvate. This reaction proceeds through an oxidative decarboxylation of the 2-oxoacid side chain of the substrate accompanied by hydroxylation of the aromatic ring and a 1,2 migration of the carboxymethyl group (Scheme 1) [1].



Scheme 1 Schematic representation of the reaction catalysed by HPPD

It is well established that this enzyme activity is involved in the catabolism of the aromatic amino acid tyrosine [2]. Humans are very sensitive to defects in tyrosine catabolism. Loss-of-function mutations in structural genes of this pathway cause several metabolic diseases, of which one, hypertyrosinaemia, was traced to a deficiency in HPPD [3]. This finding raised strong interest in the characterization of mammalian and bacterial HPPD. Thus liver HPPD has been purified and cloned from many different sources, including human, mouse, rat and pig [4–8]. In all these studies the purified enzymes were shown to contain non-haem iron as an essential cofactor for catalytic activity and to behave as homodimers of a 43–49 kDa subunit. The *Pseudomonas* enzyme is also a non-haem-iron protein, but behaves as a homotetramer of a 41 kDa subunit [9].

In contrast, there are few reports on the characterization of HPPD in plants [10–13]. In photosynthetic organisms, however, this enzyme plays a specific and crucial role. This is because the product of the reaction, homogentisate, is the aromatic precursor of all plastoquinones and tocopherols, which are essential elements of the photosynthetic electron-transfer chain and of the antioxidative systems respectively. Presumably, although this has not yet been documented, plant HPPD is also involved, as in other organisms, in the degradation of tyrosine. The importance of this enzymic activity in plants was demonstrated by the lethality of its inhibition by sulcotrione, a member of a new family of bleaching herbicides [12,13]. More recently, another new herbicide family, the benzoyl-isoxazoles, have been reported as HPPD inhibitors [14]. In both cases the bleaching effect was associated with an accumulation of the carotenoid precursor phytoene. It is believed that this effect results from an indirect inhibition of the phytoene desaturase activity, as a consequence of the depletion of the plastoquinone-cofactor pool [12,15].

Abbreviations used: HPPD, *p*-hydroxyphenylpyruvate dioxygenase; PFP, pyrophosphate:fructose-6-phosphate 1-phosphotransferase.

[1] To whom correspondence should be addressed.

The nucleotide sequence reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U87257.

Despite the importance of the prenylquinone biosynthesis pathway in plants, none of the enzyme activities involved in this pathway has yet been purified and characterized at the molecular level. We report here for the first time the subcellular localization and the purification of HPPD activity from cultured carrot cells, and the isolation of the corresponding cDNA.

MATERIALS AND METHODS

Plant material

Cultured carrot cells were grown on Murashige and Skoog medium at 25 °C, with shaking at 100 rev./min, and were subcultured every 7 days.

Preparation and fractionation of carrot cell protoplasts

Protoplasts were prepared by the method of [16] by enzymic digestion of 7-day-old cell cultures; 18 h before protoplast preparation, carrot cells were transferred to a sucrose-free medium to decrease the amount of starch within plastids. This step is critical to avoid cross-contamination of the cytosolic fraction by stromal proteins. The cells were washed twice in culture medium containing 0.5 M sorbitol, then subjected to enzymic digestion in 1 % (w/v) cellulase 'onozuka' R-10 (Yakult Honsha) and 0.1 % (w/v) pectolyase Y-23 (Kikkoman) for 30 min at 25 °C. Protoplasts were washed with 0.5 M sorbitol/1 mM $CaCl_2$/20 mM Tris/HCl (pH 7.5) and then centrifuged at 100 ***g***. This washing–centrifugation step was repeated three times to remove any traces of the digestion enzymes used for the preparation of protoplasts. Protoplasts were resuspended in the above washing buffer containing 5 mM 6-aminohexanoic acid, 1 mM benzamidine and 1 mM dithiothreitol, and gently ruptured by passing first through a 20 μm nylon mesh and subsequently through a 10 μm nylon mesh. The protoplast lysate was centrifuged successively at 900 ***g*** for 5 min, 4000 ***g*** for 5 min, and 15000 ***g*** for 20 min; the corresponding pellets, referred to as P1, P2 and P3, were resuspended in the same washing buffer as above, to yield the different organelle fractions. P1 contained the unbroken protoplasts, most of the plastids, some mitochondria and some peroxisomes; P2 contained the remaining plastids and some mitochondria; P3 contained the remaining mitochondria and peroxisomes. The 15000 ***g*** supernatant (referred to as S3) corresponded to the cytosolic fraction.

Purification of HPPD

Precipitation with streptomycin sulphate

Protoplasts were lysed osmotically by adding 2 vol. of 1 mM EDTA/1 mM dithiothreitol/5 mM 6-aminohexanoic acid/1 mM benzamidine/20 mM Tris/HCl (pH 7.5) (buffer A), and then centrifuged at 40000 ***g*** for 20 min. The supernatant was subjected to precipitation with streptomycin sulphate (1 %, w/v), and centrifuged again at 40000 ***g*** for 20 min. The remaining supernatant was used for the purification of HPPD.

Fractogel EMD DEAE 650(M) chromatography

The above protein extract (400 ml, equivalent to 1.8 g of protein) was applied to a Fractogel EMD DEAE 650 (M) column (2.6 cm × 35 cm; Merck) previously equilibrated in buffer A. The column was washed with 200 ml of buffer A and elution was performed with the following linear gradient: 0–0.1 M NaCl in buffer A (120 ml), a 60 ml wash in buffer A containing 0.1 M NaCl, 0.1–0.5 M NaCl in buffer A (580 ml), and a 150 ml wash with 1 M NaCl in buffer A (flow rate 4 ml/min, 10 ml fractions). HPPD activity was eluted as a single peak with buffer A containing 140 mM NaCl.

Phenyl-Sepharose chromatography

Fractions containing the highest HPPD activity were pooled (60 ml), raised to 2.5 M NaCl and applied to a phenyl-Sepharose CL4B (1.6 cm × 15 cm; Pharmacia) column, previously equilibrated with buffer A containing 2.5 M NaCl. The column was washed with 40 ml of equilibrating buffer and elution was performed by four successive washes with buffer A containing 1.25 M NaCl, 0.75 M NaCl, 0.25 M NaCl and no NaCl (flow rate 1 ml/min, 2 ml fractions). HPPD activity was eluted in buffer A without NaCl.

Mono Q HR5/5 chromatography

The active fractions were pooled (20 ml), and loaded on a Mono Q HR5/5 (Pharmacia) previously equilibrated with buffer A. The column was washed with 10 ml of buffer A and elution performed with the following linear gradient in buffer A: 0–0.145 M NaCl (20 ml), a 7 ml wash with buffer A containing 0.145 M NaCl, 0.145–0.180 M NaCl in buffer A in 7 ml, a 10 ml wash with buffer A containing 0.180 M NaCl, and a 20 ml wash with buffer A containing 1 M NaCl. HPPD activity was eluted at 0.180 M NaCl. The most active fractions were pooled (8 ml) and concentrated to a final volume of approx. 1.5 ml with Macrosep-10 tubes (Filtron).

Superdex 200 chromatography

The active concentrated fraction was applied to a Superdex 200 column (1.6 cm × 60 cm; Pharmacia) previously equilibrated in buffer A. Elution was performed with the equilibrating buffer at a flow rate of 1 ml/min. Fractions of 1.5 ml were collected. Fractions containing the highest HPPD activity were combined.

Electrophoretic analyses of proteins

Polypeptides were separated by SDS/PAGE [12 % (w/v) gel]. The experimental conditions for gel preparation, sample solubilization, electrophoresis and gel staining were as detailed by Chua [17].

Immunological analysis

Preparation of polyclonal antibodies specific to HPPD from *Pseudomonas fluorescens*

HPPD was purified to homogeneity from *Ps. fluorescens* sp. strain A32 by the method described by Lindstet and Odelhög [9], starting with *Ps. fluorescens* cultures grown on L-tyrosine as the sole carbon source. Antibodies directed against the purified HPPD were raised in a rabbit by standard protocols.

Immunoblotting analysis

After separation by PAGE, the proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad) by the method of Towbin et al. [18]. Membranes were incubated for 30 min in TBS [10 mM Tris/HCl (pH 7.6)/150 mM NaCl] containing 2 % (v/v) Tween-20. They were incubated for 2 h with the specific antibodies in TBS containing 0.05 % (v/v) Tween-20, and for 1 h with goat anti-(rabbit IgG)–horseradish peroxidase conjugate

(Bio-Rad). Membranes were stained for peroxidase activity, with chloronaphthol and H_2O_2 as substrates.

Amino acid sequence analysis

The purest fractions corresponding to two rounds of purification were pooled, separated by SDS/PAGE and transferred electrophoretically to a PVDF protein-sequencing membrane (Bio-Rad). Polypeptides were revealed by staining with Coomassie Blue R-250 (0.1 %) in 50 % (v/v) methanol for 5 min and destaining with several changes of 50 % (v/v) methanol/10 % (v/v) acetic acid. The stained protein band of interest was cut out and subjected to sequencing. The sequence of amino acids at the N-terminus of HPPD was determined with a Model 477 A gas–liquid-phase protein sequencer (Applied Biosystems) equipped with a Model 120 A on-line phenylthiohydantoin amino acid analyser.

Assay for HPPD

Activity was measured in a volume of 200 μl containing 100 mM Tris/HCl, pH 7.5, 50 mM ascorbate and 200 μM *p*-hydroxyphenylpyruvate. The reaction medium was incubated for 15 min at 30 °C except when otherwise stated. The reaction was stopped by the addition of 70 μl of 20 % (w/v) perchloric acid. The precipitated protein was removed by centrifugation at 15000 ***g*** for 5 min. The amount of homogentisate formed was determined by HPLC. An aliquot (50–100 μl) of the perchloric acid supernatant was injected on to a Pico Tag C_{18} (3.9 mm × 15 cm, 10 μm particle size; Millipore–Waters) column connected to an HPLC system. Buffers used for elution were as follows: A, 0.1 % (v/v) trifluoroacetic acid in distilled water; B, 0.07 % (v/v) trifluoroacetic acid in 80 % (v/v) CH_3CN. The following linear gradients were used: 0 % (100 % A) to 70 % B, 0–17 min; 70–100 % B, 17–20 min; 100 % B, 20–24 min; 100–0 % B, 24–28 min; flow rate 1 ml/min. Homogentisate was detected by measuring the UV absorbance at 288 nm. Quantification of homogentisate was performed by measuring peak areas with 450-MT2 data-capture software (Kontron). Areas were transformed to nmol of homogentisate by comparison with a standard curve.

Measurement of marker enzyme activities

Except where otherwise stated, enzymes were assayed spectrophotometrically in 1 ml reaction volumes. Triton X-100 (0.1 %, w/v) was added to each reaction mixture to ensure the rupture of all the membranes surrounding the organelles. The coupled enzyme systems (Boehringer) were determined as not being rate-limiting, and the activity of each marker enzyme in carrot cellular extract was strictly dependent on the presence of all necessary substrates and cofactors. The marker enzyme activities were linear with respect to time and the amount of extract assayed.

Pyrophosphate:fructose-6-phosphate 1-phosphotransferase (PFP; EC 2.7.1.90) [19] was assayed in 100 mM Hepes/NaOH (pH 7.8)/1 mM $MgCl_2$/5 mM fructose 6-phosphate/150 μM NADH containing 0.2 unit of aldolase, 10 units of triosephosphate isomerase and 0.5 unit of glyceraldehyde-3-phosphate dehydrogenase. The samples were preincubated for 2 min with 2 μM fructose 2,6-bisphosphate (to activate PFP) and 5 mM NaF (to inhibit any pyrophosphatase activity without affecting the PFP activity), and the reaction was initiated with 1 mM pyrophosphate. Activity was assayed spectrophotometrically at 340 nm by measuring the coupled oxidation of NADH.

ADP-glucose pyrophosphorylase (EC 2.7.7.27) was assayed in 50 mM Tricine/NaOH (pH 8.0)/5 mM $MgCl_2$/2 mM dithiothreitol/5 mM NaF/1 mM ADP-glucose/5 mM glycerate 3-phosphate (to activate ADP-glucose pyrophosphorylase)/10 μM glucose 1,6-bisphosphate (to activate phosphoglucomutase)/500 μM $NADP^+$/1 mM PP_i containing 0.8 unit of phosphoglucomutase and 0.7 unit of glucose-6-phosphate dehydrogenase [20]. Activity was assayed spectrophotometrically at 340 nm by measuring the coupled reduction of $NADP^+$.

Fumarase (EC 4.2.1.2) was assayed by the method of Hill and Bradshaw [21] by following the appearance of fumarate at 240 nm from reaction assays containing 50 mM Tricine, pH 7.5, and 50 mM malate.

Catalase (EC 1.11.1.6) was assayed by the method of Van Ginkel and Brown [22] by following the release of oxygen with a Clark-type O_2 electrode purchased from Hansatech (King's Lynn, Norfolk, U.K.). The reaction medium contained 20 mM Tris/HCl, pH 7.5, 5 mM H_2O_2 and 0.1 % (w/v) Triton X-100. The reaction medium was made free of oxygen by bubbling with argon before starting the reaction.

Isolation of a full-length HPPD cDNA

A keyword search of the National Agricultural Library Database identified an *Arabidopsis* EST clone that contained an open reading frame with a good similarity to the human HPPD. This clone, 96B13T7 (GenBank accession no. T20952), was obtained from the *Arabidopsis* Stock Center (The Ohio State University, Columbus, OH, U.S.A.) and sequenced. Its open reading frame coded for 75 amino acids exhibiting a high similarity to the C-terminal sequence of the mammalian HPPD. However, the position at which the similarity starts suggested that clone 96B13T7 was not full-length. To obtain a full-length clone of carrot HPPD, the insert of 96B13T7 was radioactively labelled and used as a probe to screen a carrot cell cDNA library, constructed in λ ZAPII (Stratagene). Plaque screening was performed in accordance with the manufacturer's instructions; 250000 clones were screened, yielding 12 positive clones. The clone containing the longest insert was analysed by DNA sequence analysis, which was performed on both strands with a Prism Kit with fluorescent dideoxynucleotides, *Taq* DNA polymerase (Applied Biosystems) and T3 and T7 universal primers. In addition, specific oligonucleotide primers were used for further sequencing. Gene Works 2.4 and PCGENE (Intelligenetics) sofware were used for sequence analyses.

Northern-blot analysis

mRNAs were isolated from carrot cells with the 'Straight A's mRNA isolation system' (TEBU). The mRNA species (4 μg) were denatured [1 h at 50 °C in 10 mM sodium phosphate (pH 7)/2 % (v/v) DMSO/1.08 M glyoxal] and separated by 1 % (w/v) agarose-gel electrophoresis. The mRNA species were then transferred to a nylon membrane (Nitran) by the method of Thomas [23]. The resulting blots were subjected to hybridization with the corresponding ^{32}P-labelled cDNA probe coding for HPPD.

Construction of S-Tag HPPD Car 13 fusion in the expression vector pET29a

The pET29-HPPD Car 13 plasmid coding for a carrot HPPD fusion protein was constructed via site-directed mutagenesis with PCR amplification of the entire carrot HPPD cDNA. The following oligonucleotides were used: Pr1, 5′-GCGTGGAAACC-AAACCATGGGGAAAAAACAATCGG-3′, which introduces an *Nco*I restriction site containing the ATG translation-initiation codon (underlined); and Pr2, 5′-GGAATTTTTTTTG-

TCGACTGGACTCTC-3′, which is complementary to the 3′ end of the cDNA and introduces a *Sal*-I restriction site (underlined) 7 bp after the TGA stop codon. PCR was performed for 30 cycles including 1 min of denaturation at 95 °C, 1 min of annealing at 58 °C and 2 min of DNA elongation at 72 °C driven by the *Pwo* DNA polymerase (Boehringer). The PCR DNA fragment was subcloned into the pET29a(+) vector (Novagen) digested by *Nco*I–*Sal*I restriction enzymes. This oriented cloning put the carrot HPPD cDNA under the control of the T7 polymerase promoter. The DNA insert was sequenced at both strands to ensure that no mutation had been introduced during the course of PCR amplification.

Expression and purification of recombinant carrot HPPD

Escherichia coli BL21(DE3)pLysS cells expressing the pET29-HPPD Car 13 plasmid were grown at 37 °C in 500 ml of Luria–Bertani broth medium supplemented with 100 μg/ml carbenicillin. Isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM when bacterial growth was equivalent to a D_{600} of 0.6. The cells were further grown for 3 h at 30 °C.

The cells were harvested by centrifugation; the pellet was resuspended in 10 ml of buffer A and sonicated with a Vibra-cell disruptor (Sonics and Materials, Danbury, CT, U.S.A.) (100 pulses every 3 s on power setting 5). The crude extract was centrifuged at 35000 ***g*** for 30 min to yield a cell-free supernatant. The soluble protein extract containing the recombinant carrot HPPD fused to the S-Tag leader sequence was desalted on a PD10 Sephadex G-25 (M) column (Pharmacia) equilibrated in the following buffer: 20 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.1 % Triton X-100. This solution was then incubated with S-Protein-Agarose® (Novagen) in batch processing for 1 h at 25 °C with gentle shaking to ensure optimal binding of the fusion protein to the matrix. Purification of the recombinant protein by cleavage of the S-Tag leader sequence was achieved with biotinylated thrombin in accordance with the manufacturer's instructions (Novagen). The sequence of amino acids at the N-terminus of the purified protein was determined and found to be identical with those of the predicted carrot HPPD.

Preparation of antibodies against the recombinant carrot HPPD

Antiserum against the carrot HPPD was raised as described above for the *Ps. fluorescens* HPPD. Antibodies were further purified by 40 %-$(NH_4)_2SO_4$ precipitation and anion-exchange column chromatography (DEAE trisacryl M+; Sepracore), and then by affinity chromatography with purified recombinant carrot HPPD (1 mg) bound to CNBr-activated Sepharose 4B (Pharmacia) in accordance with the protocol recommended by the manufacturer. Dilution of this affinity-purified antibody solution to 1/2000 allowed the detection of 0.5 ng of purified recombinant carrot HPPD, and was used for the immuno-detection of carrot HPPD in different carrot cell extracts.

RESULTS

HPPD activity of cultured carrot cells

To avoid the use of ^{14}C-labelled substrate a non-radioactive assay for HPPD activity, based on a determination by HPLC and UV absorbance at 288 nm of the amount of homogentisate formed, was used in this study. Homogentisate was identified by comparison with authentic standard. Among all the plant extracts assayed, which included pea, maize, spinach and lettuce, cultured carrot cells revealed the highest HPPD activity. A time course study showed that the accumulation of homogentisate was linear with protein amount and time for at least 15 min in the presence of 0.3 mg of total carrot cell extract proteins and 200 μM *p*-hydroxyphenylpyruvate (Figures 1A and 1B). The specific activity of the soluble protein extract was in the range 0.6–0.9 nmol of homogentisate formed/min per mg of protein.

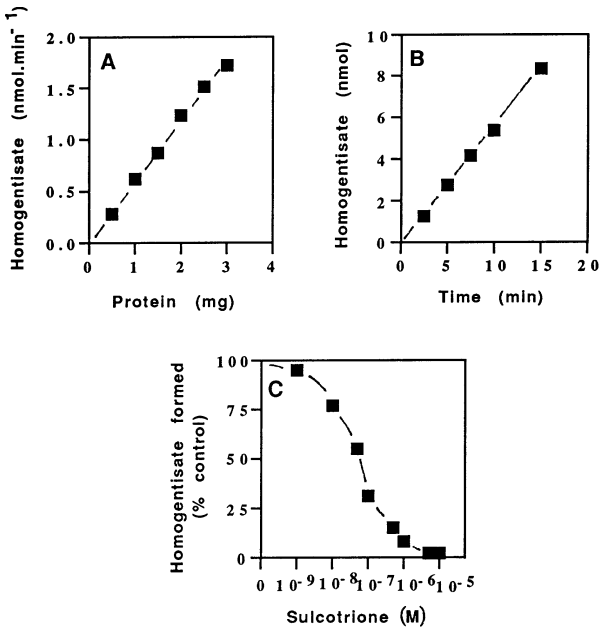


Figure 1 Kinetic parameters of carrot cell HPPD activity

Dependence of HPPD activity on the concentration of protein (**A**) and on time (**B**), and inhibition of the carrot-cell HPPD by sulcotrione (**C**). Assays were run as described in the Materials and methods section with the soluble fraction obtained after streptomycin sulphate precipitation. The *p*-hydroxyphenylpyruvate concentration used was 200 μM.

The carrot cell HPPD activity was found to be highly sensitive to inhibition by the herbicide sulcotrione (IC_{50} approx. 60 nM; Figure 1C), as reported for other plant enzymes [12,13].

Intracellular localization of HPPD activity

To improve our knowledge of prenylquinone biosynthesis in plants, the subcellular localization of HPPD activity in carrot cells was determined. This was investigated by using protoplasts isolated from carrot cells. Intact protoplasts were fractionated by gentle rupture through a fine nylon mesh; fractions enriched in plastid, mitochondrial, peroxisomal and cytosolic proteins were prepared by differential centrifugation as described in the Materials and methods section. These fractions were assayed for HPPD activity, their purity being assessed by measurement of selected subcellular marker enzyme activities. As shown in Table 1, most plastidial (73 %), mitochondrial (87 %) and peroxisomal (76 %) marker activities were recovered in the pellet fractions (P1, P2 and P3) described in the Materials and methods section. In contrast, most of the cytosolic marker activity (84 %) and most of the HPPD activity (93 %) were recovered in the 15000 ***g***-supernatant fraction (S3). This fraction also contained 20 %, 3 % and 29 % of the plastid, mitochondrial and peroxisomal marker enzyme activities respectively. Table 1 also shows that the HPPD activity found in the three pellets was of the same order of magnitude as the respective level of contamination of each of these pellets by the cytosolic marker enzyme activity (PFP). It thus seems very unlikely that plastids and mitochondria contain significant HPPD activity. Furthermore, starting from Percoll-

Table 1 Subcellular localization of HPPD activity in carrot cell protoplasts

HPPD activity was assayed in broken carrot-cell protoplasts (protoplast extract; 100 mg of protein), 900 ***g*** pellet (P1), 4000 ***g*** pellet (P2), 15000 ***g*** pellet (P3) and 15000 ***g*** supernatant (S3). Marker enzyme activities are also indicated. The marker enzymes were as follows: cytosol, PFP; plastids, ADP-glucose pyrophosphorylase (ADP-GPP); mitochondria, fumarase; peroxisomes, catalase. The results presented for distribution in supernatant and pellets and for recovery are expressed as percentages of total activity recovered, and are the means ± S.D. for three different experimental determinations.

	Enzyme activity (μmol/min)				
Distribution	PFP	ADP-GPP	Fumarase	Catalase	HPPD
Protoplast extract	11	0.6	31	4290	6.7×10^{-3}
	Enzyme activity (% of total)				
Distribution	PFP	ADP-GPP	Fumarase	Catalase	HPPD
P1	12 ± 2	63 ± 4	45 ± 4	44 ± 3	11 ± 2
P2	0.5	8 ± 1	12 ± 1	4 ± 1	0.4
P3	0.7	1.5	30 ± 2	28 ± 2	–
S3	84 ± 6	20 ± 2	3	29 ± 2	93 ± 4
Recovery	97 ± 8	92 ± 7	88 ± 7	106 ± 8	104 ± 6

Table 2 Partial purification of HPPD from carrot cells

One unit of HPPD corresponds to 1 nmol of homogentisate formed during 15 min under the standard conditions described in the Materials and methods section.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Total extract	2733	16824	6	100
Streptomycin sulphate	1858	16038	9	95
DEAE	317	4256	13	25
Phenyl-Sepharose CL4B	32	553	17	3.3
Mono Q HR5/5	5	304	61	1.8
Superdex 200	0.5	45	91	0.6

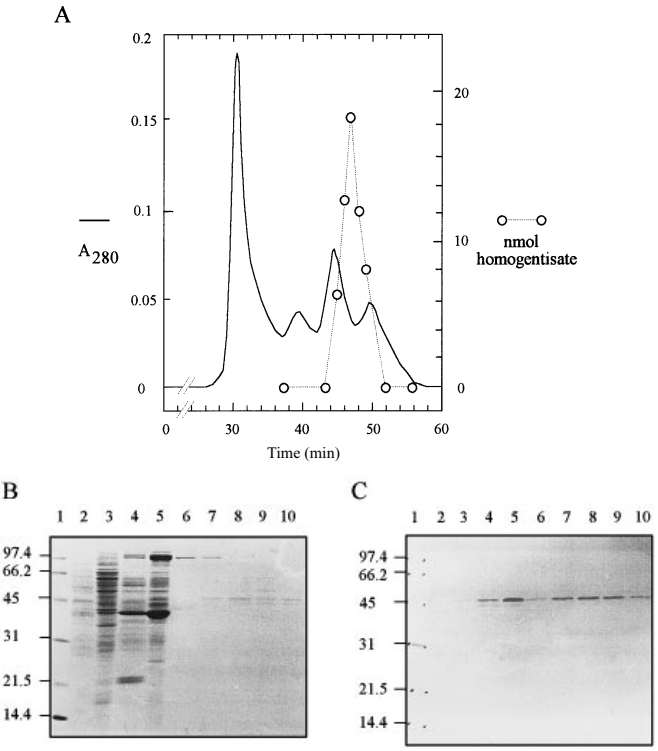


Figure 2 Documentation of the purification procedure for HPPD activity

Elution profile of HPPD activity from the Superdex 200 gel-filtration column (**A**), and SDS/PAGE analysis of purification procedure (**B**) and the corresponding immunoblot analysis (**C**). HPPD activity is expressed as nmol of homogentisate formed per 15 min by the total fraction. Polypeptides were separated on a 12% (w/v)-polyacrylamide gel under denaturing conditions and stained with Coomassie Blue R-250 (**B**) or analysed by Western blotting after incubation with polyclonal rabbit antibodies raised against the purified *Ps. fluorescens* HPPD (**C**). Lane 1, molecular mass markers (molecular masses indicated at the left in kDa); lane 2, cellular extract after streptomycin sulphate precipitation (100 μg); lane 3, DEAE pool (100 μg); lane 4, phenyl-Sepharose pool (100 μg); lane 5, Mono-Q HR5/5 pool (100 μg). Lanes 6–10 correspond to fractions 45–49 of the Superdex 200 elution profile shown in (**A**) respectively (100 μl).

purified plastids of carrot cells, we were unable to detect any HPPD activity by the enzymic assay described in the Materials and methods section (results not shown). From these results it is clear that in cultured carrot cells most, if not all, of the HPPD activity measured is extraplastidial. Moreover, although contamination of the 15000 ***g*** supernatant (S3), which corresponds to the cytosolic fraction, by peroxisomal proteins was significant (29% of total peroxisomal proteins was recovered in the cytosolic fraction), the HPPD activity measured in this fraction (93% of the total activity) could not be ascribed to the presence of peroxisomal contaminants. Indeed, the P3 pellet, which contained a similar amount of peroxisomal proteins (29%) to that in the S3 supernatant, was completely devoid of HPPD activity. We thus conclude that the carrot cell HPPD activity that we measure is associated with the cytosolic fraction.

Purification of HPPD

HPPD activity was partly purified by a sequence of chromatographic steps including ion-exchange chromatography, hydrophobic interaction chromatography and gel filtration as described in the Materials and methods section. Table 2 summarizes the purification procedure.

The major problem encountered during the purification was a significant loss of enzyme activity. Although the HPPD activity was stable after the streptomycin sulphate precipitation step, a large loss of activity occurred after each subsequent chromatographic step and also during storage of the active fractions, whatever the precautions taken. For example, all the buffers used for the chromatographic steps contained dithiothreitol and protease inhibitors, and were purged with argon to prevent possible oxidation of the enzyme. In addition, purifications were performed in the presence of 20% glycerol, the enzyme substrate *p*-hydroxyphenylpyruvate, or of a substrate analogue (2-hydroxyphenylpyruvate) without any significant improvement in enzyme stability and recovery. Finally, all attempts to restore the enzyme activity by adding ferrous iron to the chromatographic buffers or to the assay medium were unsuccessful.

An SDS/PAGE analysis illustrating the progress of purification is shown in Figure 2. The Superdex 200 gel-filtration chromatographic step was the last purification step where the enzyme retained some activity. As shown in Figure 2(A), HPPD was eluted from this column as a single peak of activity; and this chromatographic step allowed the separation of most contaminant polypeptides (Figure 2B). Lanes 6–10 in Figure 2(B) corresponded to fractions 45–49 of the Superdex 200 elution

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cacgaggattgagtgcctcgattaccagaatataccatacttgcttgcctagtacactagttatatttacacata      75
                *
aacacacagcgtggaaaccaaaaaATGGGGAAAAAACAATCGGAAGCTGAAATTCTCTCAAGCAATTCATCAAAC     150
                      M  G  K  K  Q  S  E  A  E  I  L  S  S  N  S  S  N
ACCTCTCCTGCAACATTCAAGCTGGTCGGTTTCAACAACTTCGTCCGCGCCAACCCCAAGTCCGATCACTTCGCC     225
T  S  P  A  T  F  K  L  V  G  F  N  N  F  V  R  A  N  P  K  S  D  H  F  A
GTGAAGCGGTTCCACCACATTGAGTTCTGGTGCGGCGACGCCACCAACACGTCGCGGCGGTTCTCGTGGGGCCTC     300
V  K  R  F  H  H  I  E  F  W  C  G  D  A  T  N  T  S  R  R  F  S  W  G  L
GGCATGCCTTTGGTGGCGAAATCGGATCTCTCTACTGGAAACTCTGTTCACGCTTCTTATCTTGTTCGCTCGGCG     375
G  M  P  L  V  A  K  S  D  L  S  T  G  N  S  V  H  A  S  Y  L  V  R  S  A
AATCTCAGTTTCGTCTTCACCGCTCCTTACTCTCCGTCCACGACCACTTCCTCTGGTTCAGCTGCCATCCCGTCT     450
N  L  S  F  V  F  T  A  P  Y  S  P  S  T  T  T  S  S  G  S  A  A  I  P  S
TTTTCGGCATCGGGTTTTCACTCTTTTGCGGCCAAACACGGCCTTGCTGTTCGGGCTATTGCTCTTGAAGTTGCT     525
F  S  A  S  G  F  H  S  F  A  A  K  H  G  L  A  V  R  A  I  A  L  E  V  A
GACGTGGCTGCTGCGTTTGAGGCCAGTGTTGCGCGTGGGGCCAGGCCGGCTTCGGCTCCTGTTGAATTGGACGAC     600
D  V  A  A  A  F  E  A  S  V  A  R  G  A  R  P  A  S  A  P  V  E  L  D  D
CAGGCGTGGTTGGCTGAGGTGGAGTTGTACGGACATGTGGTCTTGAGGTTTGTTAGTTTTGGGAGGGAGGAGGGT     675
Q  A  W  L  A  E  V  E  L  Y  G  D  V  V  L  R  F  V  S  F  G  R  E  E  G
TTGTTTTTGCCTGGATTCGAGGCGGTGGAGGGGACGGCGTCGTTTCCGGATTTGGATTATGGAATTAGAAGACTT     750
L  F  L  P  G  F  E  A  V  E  G  T  A  S  F  P  D  L  D  Y  G  I  R  R  L
GATCATGCGGTGGGGAATGTTACCGAGTTGGGGCCTGTGGTGGAGTATATTAAAGGGTTTACGGGGTTTCATGAA     825
D  H  A  V  G  N  V  T  E  L  G  P  V  V  E  Y  I  K  G  F  T  G  F  H  E
TTTGCGGAGTTTACAGCGGAGGATGTGGGGACTTTGGAGAGTGGGTTGAATTCGGTGGTGTTGGCGAATAATGAG     900
F  A  E  F  T  A  E  D  V  G  T  L  E  S  G  L  N  S  V  V  L  A  N  N  E
GAGATGGTTCTGTTGCCCTTGAATGAGCCTGTGTATGGGACCAAGAGGAAGAGTCAGATACAGACTTACTTGGAG     975
E  M  V  L  L  P  L  N  E  P  V  Y  G  T  K  R  K  S  Q  I  Q  T  Y  L  E
CACAATGAAGGGGCTGGAGTGCAGCATTTGGCTTAGTGAGTGAGGATATTTTTAGGACTTTAAGGGAGATGAGG    1050
H  N  E  G  A  G  V  Q  H  L  A  L  V  S  E  D  I  F  R  T  L  R  E  M  R
AAGAGGAGTTGCCTTGGTGGTTTTGAGTTTATGCCTTCGCCACCGCCTACGTATTACAAGAATTTGAAGAATAGG    1125
K  R  S  C  L  G  G  F  E  F  M  P  S  P  P  P  T  Y  Y  K  N  L  K  N  R
GTCGGGGATGTGTTGAGTGATGAACAGATCAAGGAGTGTGAAGATTTGGGGATTTTGGTGGATAGGGATGATCAG    1200
V  G  D  V  L  S  D  E  Q  I  K  E  C  E  D  L  G  I  L  V  D  R  D  D  Q
GGTACATTGCTTCAAATCTTTACCAAGCCTGTAGGTGACAGGCCTACCTTATTCATAGAGATCATTCAGAGGGTA    1275
G  T  L  L  Q  I  F  T  K  P  V  G  D  R  P  T  L  F  I  E  I  I  Q  R  V
GGGTGCATGCTCAAGGACGATGCAGGGCAGATGCACCAGAAGGGCGGGTGCGGAGGATTTGGGAAGGGGAACTTC    1350
G  C  M  L  K  D  D  A  G  Q  M  Y  Q  K  G  G  C  G  G  F  G  K  G  N  F
TCAGAGCTGTTCAAGTCCATCGAAGAATATGAAAAAACACTTGAAGCTAAACAAATCACTGGATCTGCTGCTGCA    1425
S  E  L  F  K  S  I  E  E  Y  E  X  T  L  E  A  K  Q  I  T  G  S  A  A  A
TGAgagtccagttaacacaaaaattcctcaagtgttttacattatatgactgcaaatgtgtaacctccgtgtat     1500
*
aatataatgtttttaagccaaaccagcattcacataacctgctttcctcaggtatgcacaaaagatggattgaca     1575

ttgtataacttgcaagtggtatgttattcatctttctaatttacaaagagaaatataa aataaagtactaagct     1650

tttacctagctcaaaaaaaaaaaaaaaaaaaaaa                                            1684
```

Figure 3 Nucleotide and predicted amino acid sequences of the cDNA encoding HPPD from carrot cells (HPPD Car 13 clone)

The coding sequence is indicated with capital letters, and the non-coding sequence with lower-case letters. Nucleotides are numbered at the right. The first in-frame ATG codon is in bold and the corresponding stop codon is marked with an asterisk. The amino acids corresponding to the N-terminal sequence of the purified native carrot HPPD are in bold. The open reading frame extends for 1324 bp and encodes a predicted polypeptide of 442 amino acid residues with a molecular mass of 48094 Da.

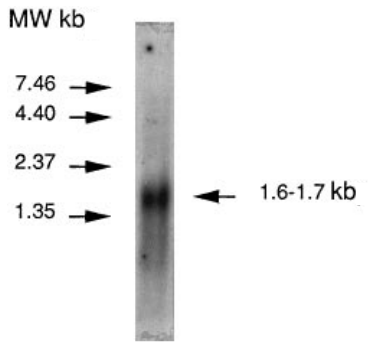

Figure 4 Northern-blot analysis of carrot cell mRNA

mRNA species were isolated from carrot cells with the 'Straight A's mRNA isolation system' (TEBU). The mRNA (4 μg) was denatured [1 h at 50 °C in 10 mM NaH_2PO_4 (pH 7)/2% (v/v) DMSO/1.08 M glyoxal] and separated by 1% (w/v)-agarose-gel electrophoresis. They were then transferred to nylon membrane (Nitran) by the method of Thomas [23]. The resulting blots were subjected to hybridization with the corresponding ^{32}P-labelled cDNA probe coding for HPPD. The carrot HPPD cDNA hybridized with a single transcript of 1.6–1.7 kb.

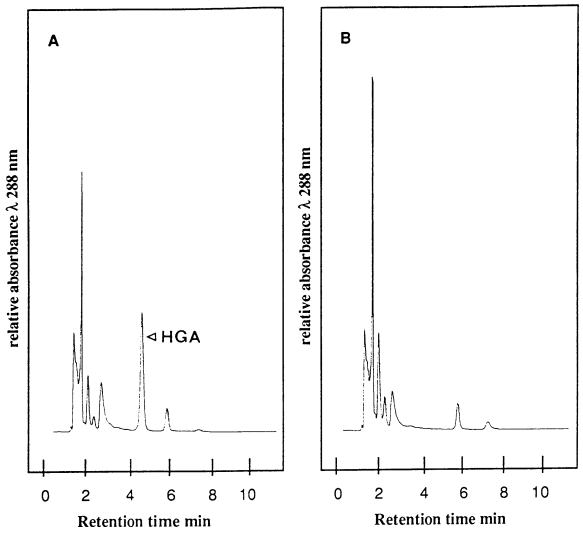

Figure 5 Functional characterization of HPPD Car 13 cDNA

Representative HPLC chromatograms showing the synthesis of homogentisate by *E. coli* BL21 cell extract harbouring the plasmid pET29-HPPD Car 13 (**A**) and its inhibition by 0.5 μM sulcotrione (**B**). Assays were run in presence of 200 μM *p*-hydroxyphenylpyruvate and 0.5 μl (15 μg of proteins) of *E. coli* BL21 cell extract as described in the Materials and methods section. The specific activity of the cellular extract was approx. 50 nmol of homogentisate formed/min per mg of proteins. Note that *E. coli* BL21 cells have no endogenous HPPD activity. Abbreviation: HGA, homogentisate.

profile in Figure 2(A). Four polypeptides were detected in the purest fraction (Figure 2B, lane 8). Of these, only two followed exactly the elution profile of enzyme activity: a major polypeptide of 45–46 kDa and a minor one of 43–44 kDa. To determine which of these two polypeptides corresponded to the carrot HPPD, we performed an immunoblotting analysis with polyclonal rabbit antibodies raised against the *Ps. fluorescens* HPPD that had previously been purified to homogeneity by the method of [9]. As shown in Figure 2(C), the antiserum cross-reacted specifically with the major polypeptide of 45–46 kDa. This polypeptide accumulated during the purification procedure, but because of its low abundance it was not detected in the total cellular extract. From all these results, it seems likely that this 45–46 kDa polypeptide corresponds to the carrot cell HPPD. The first 20 N-terminal amino acids of this polypeptide were identified as VRANPKSDHFAVKRFHHIEF.

Isolation of cDNA clones encoding carrot HPPD

To characterize the carrot HPPD further at the molecular level, and to confirm that the 45 kDa polypeptide is the carrot HPPD, we isolated a full-length HPPD cDNA from a carrot cell cDNA library. This carrot HPPD clone was isolated with an *Arabidopsis* EST as a nucleotide probe. This EST was putatively identified as a HPPD cDNA clone because of the good similarity of its open reading frame to the C-terminal sequence of mammalian HPPD. The complete nucleotide sequence of the longest isolated cDNA (HPPD-Car 13) is shown in Figure 3 together with the deduced amino acid sequence. HPPD-Car 13 is a 1684 bp cDNA including one large open reading frame of 1324 bp. The first in-frame ATG, occurring at nt 100, initiates this 1324 bp open reading frame, which encodes a predicted polypeptide of 442 amino acid residues with a molecular mass of 48094 Da. The nucleotide sequence around the first ATG codon, AAAATGGGG (positions 97–105) quite closely matches the plant consensus translation initiation motif, AACAAUGGC [24]. Furthermore, the presence of an in-frame nonsense TGA codon 90 bp upstream from the first ATG, the molecular mass of the predicted polypeptide, and the fact that Northern-blot analysis reveals a single transcript of 1.6–1.7 kbp (Figure 4), confirm that HPPD-Car 13 is full length.

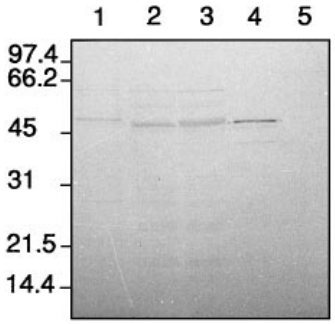

Figure 6 Immunodetection of the native carrot HPPD by using immunopurified carrot HPPD antibodies

Polypeptides were separated by SDS/PAGE [12% (w/v) gel] under denaturing conditions and analysed by Western blotting after incubation with immunopurified polyclonal rabbit antibodies (1:2000 dilution) raised against the purified recombinant carrot HPPD. Lane 1, cellular extract obtained by direct grinding of fresh carrot cells in liquid nitrogen (60 μg); lane 2, cellular extract obtained by osmotic rupture of carrot cell protoplasts (60 μg); lane 3, cytosolic fraction (S3) (60 μg); lane 4, Mono-Q HR5/5 pool of the partly purified native carrot HPPD (20 μg); lane 5, Percoll-purified plastids (60 μg). The positions of molecular-mass markers (in kDa) are shown at the left.

Functional characterization of HPPD-Car 13

E. coli BL21 cells harbouring the plasmid pET29-HPPD Car 13 produce a characteristic brownish pigment that has been previously observed by other groups cloning HPPD from different origins. This pigment is a product of the oxidation and polymerization of homogentisate synthesized by the recombinant HPPD [25–27]. Accumulation of this pigment was prevented by the addition of sulcotrione in the culture medium (results not shown). Furthermore, the activity of the carrot recombinant HPPD was measured in the soluble protein extract of *E. coli* BL21 cells expressing the pET29-HPPD Car 13 plasmid (Figure 5). The specific activity of this protein extract was 50 nmol of homogentisate formed/min per mg of protein. As with the native HPPD, the activity of the recombinant HPPD was inhibited by sulcotrione (Figure 5B).

Immunodetection of the carrot HPPD within carrot cell extracts with immunopurified carrot HPPD antibodies

The recombinant S-Tag fusion-carrot HPPD produced by *E. coli* BL21 cells expressing pET29a-HPPD Car13 plasmid was purified from the soluble protein extract by the affinity-chromatography protocol recommended by the manufacturer (see the Materials and methods section). Pure protein (500 μg) was used to produce rabbit polyclonal antibodies, which were further immunopurified by affinity chromatography with purified recombinant carrot HPPD bound to CNBr-activated Sepharose 4B. A 1:2000 dilution of the immunopurified antibodies specifically revealed the carrot HPPD (Figure 6). The molecular mass of the polypeptide immunodetected differed slightly depending on the method of preparation of the carrot cellular extract. The molecular mass of the polypeptide detected in a crude protoplast lysate (lane 2), which exactly corresponded to the molecular mass of the purified native HPPD (lane 4), was slightly lower than when the crude extract was prepared by grinding fresh carrot cells in liquid nitrogen (lane 1). This result indicates that proteolysis of HPPD occurs during the preparation of the carrot cellular extract (note that this proteolysis also occurs during the purification of carrot HPPD even when starting with the crude extract prepared by grinding fresh carrot cells in liquid nitrogen). Figure 6 also shows that, in agreement with the cytosolic localization of its enzymic activity, HPPD was immunodetected in the cytosol (lane 3), but not in Percoll-purified plastids of carrot cells (lane 5).

DISCUSSION

Despite its potential importance in chloroplast function and the fact that it is the molecular target of new potent herbicide families, there are few reports on HPPD activity in plants. This is presumably due to its low abundance in plant tissues and to the difficulty of measuring its enzymic activity. The results presented in this study allow for the first time the molecular characterization of a plant HPPD and its subcellular compartmentation.

In all previous studies, HPPD activity has been monitored through radioactive assays, i.e. through the release of $^{14}CO_2$ [12] and/or the incorporation of ^{14}C or ^{3}H into homogentisate or prenylquinones [10,11,13]. In this study, plant HPPD activity was assayed directly through the determination of homogentisate formed, by HPLC, without any requirement for radiolabelled substrate.

Among all the plant species screened for HPPD activity (pea, maize, spinach and lettuce), carrot cell cultures exhibited the highest level of activity. Despite its instability, carrot cell HPPD was purified to nearly complete homogeneity, and its identification was further confirmed by an immunological analysis with an antiserum raised against the HPPD of *Ps. fluorescens*. These results strongly suggest that the carrot cell HPPD activity that we measure is supported by a 45–46 kDa polypeptide. Fractionation of carrot cell protoplasts clearly indicates that this carrot HPPD is associated with the cytosol, because its activity specifically follows that of the cytosolic marker enzyme PFP, but not those of the plastidial, peroxisomal or mitochondrial marker enzymes.

The elution profile of enzyme activity from the Superdex S 200 gel-filtration column revealed that this enzyme behaves as a homodimer; thus in this respect it resembles the corresponding mammalian enzyme, which is also a homodimer of 45–49 kDa subunits [4–6]. In contrast, the *Pseudomonas* enzyme exists as a homotetramer [9]. As with all the HPPDs examined so far [12,13,28], the carrot cell activity was found to be very sensitive to sulcotrione (IC_{50} approx. 60 nM).

Detailed characterization of plant HPPD will require large quantities of pure and active enzyme. To overcome the diffficulties encountered during the purification of the native carrot HPPD to homogenity, and to confirm that the 45–46 kDa polypeptide that we have purifed effectively supports the carrot HPPD activity, we isolated a carrot HPPD cDNA. A comparison of the predicted protein sequence of this cDNA with those compiled in the GenBank database showed low but significant similarity with HPPD from *Streptomyces avermitilis* [25] (29% identity), *Homo sapiens* [8] (28% identity), *Caenorhabditis elegans* [29] (27% identity), *Coccidioides immitis* [26] (22% identity), *Schewanella colwelliana* [30] (22% identity) and *Synechocystis sp.* (accession number slr 0090) (14% identity). Specific boxes of similarity are concentrated near the C-terminus of the proteins (Figure 7). As previously observed from a comparison of mammalian, fungal, nematode and bacterial HPPD sequences [8,25–27], plant and cyanobacteria HPPDs also have highly conserved regions at their C-termini. This strongly suggests that this domain has a functional role in the catalytic process, perhaps providing the amino acid residues involved in the binding of the iron atom and/or the substrate. Indeed, two aspartic residues, two glutamic residues, two histidine residues and two tyrosine residues, which all correspond to potential ligands for a non-haem iron, can be

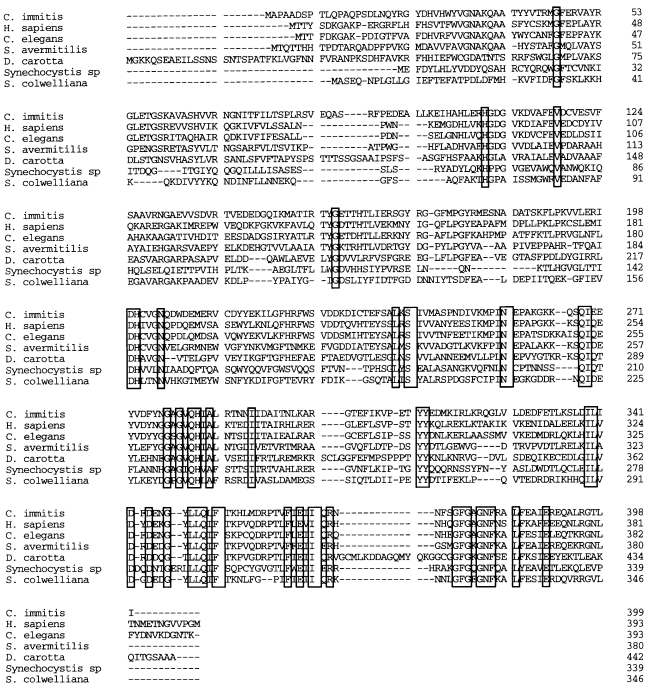

Figure 7 Amino acid sequence comparison between HPPDs of different origins

Mammalian, *Homo sapiens* [8]; fungus, *Coccidioides immitis* [26]; nematode, *Caenorhabditis elegans* [29]; bacteria, *Streptomyces avermitilis* [25], *Schewanella colwelliana* [30]; cyanobacteria, *Synechocystis* sp. (CyanoBase, the genome data base for *Synechocystis* sp. strain PCC5603, accession number slr0090). Conserved regions are shaded. Dashes indicate gaps introduced to maximize alignment.

found at conserved C-terminal positions in the seven proteins. Site-directed mutagenesis will help us to identify the residues that bind the iron, and also those involved in substrate binding. A common feature of all these HPPD is a relatively high hydrophilicity, confirming the soluble nature of the proteins. Surprisingly, the lowest similarity between all the different HPPDs and the plant HPPD was found with the *Synechocystis* protein.

Car 13 cDNA was confirmed as an HPPD cDNA by enzyme-activity measurement of the protein expressed in *E. coli*. *E. coli* BL21 cells are completely devoid of endogenous HPPD activity. In contrast, the transformed cells harbouring the plasmid pET29-HPPD Car 13 exhibit a high level of HPPD activity. As with the native enzyme, the recombinant carrot HPPD was found to be highly sensitive to sulcotrione inhibitor.

The presence, in the predicted peptide encoded by the carrot cDNA, of the 20-residue sequence determined by micro-sequencing of the native enzyme confirms that the 45–46 kDa polypeptide that we have purified supports the carrot HPPD activity. Surprisingly, this N-terminal sequence was located 30 residues downstream from the first in-frame methionine. Because the carrot HPPD activity was clearly located in the cytosol, the existence of a signal sequence suggested by this 5′ extension was quite unexpected. Furthermore, by using the program PSORT we were unable to determine clearly the presence of an N-terminal signal peptide from the complete sequence. In fact, our results indicate that during the process of purification an N-terminal proteolysis of HPPD occurs, thus explaining the difference in N-terminal sequence between the purified protein and the predicted peptide sequence encoded by the cDNA. Indeed, by using the immunopurified antibodies raised against the pure recombinant carrot HPPD, we were able to detect, in a crude extract prepared by grinding fresh carrot cells in liquid nitrogen, a 48 kDa polypeptide corresponding to the entire carrot HPPD. Moreover, in agreement with the cytosolic localization of its enzymic activity, HPPD was immunodetected in the cytosolic fraction of carrot cells, but not in Percoll-purified plastids. All these results strongly suggest that the cDNA that we have cloned encodes a cytosolic form of HPPD. To our knowledge this is the first molecular characterization of a plant HPPD. During the preparation of this manuscript a report appeared describing the partial purification of maize HPPD [31]. In contrast with our observations that the carrot HPPD is a homodimer of 48 kDa subunits, which in this respect resembles the mammalian HPPD, the maize HPPD activity was reported to co-elute with a monomeric protein of 43 kDa.

In plants, HPPD is involved in the prenylquinone biosynthetic pathway by providing homogentisate, the aromatic precursor of all prenylquinones. Tocopherols and plastoquinones are then synthesized by a series of prenyltransferases, methyltransferases and cyclase and quinol oxidase reactions that are all associated with the inner envelope membrane of the chloroplast [32]. However, in plants, as in other organisms, HPPD is also implicated in the degradation process of the aromatic amino acid tyrosine [33,34]. The occurrence of these two different, and unrelated, anabolic and catabolic processes involving HPPD raises the question of the existence of different isoforms of HPPD. So far, the only study on the localization of HPPD in plant cells reports the presence of HPPD activity in both chloroplasts and peroxisomes isolated from spinach [10]. According to those authors, the chloroplastic isoform would be involved in the biosynthesis of prenylquinones, and the peroxisomal isoform in the degradation of tyrosine.

The existence of a cytosolic HPPD in carrot cells, demonstrated by the present study, seems at first sight difficult to reconcile with the situation in spinach described by Fiedler et al. [10]. However, the possible presence of a cytosolic HPPD activity in spinach cells was not tested by those authors. In contrast, the very low level of HPPD activity found in spinach chloroplasts might explain our inability to measure and immunodetect a plastidial isoform of this enzyme in carrot cells. Alternately, this putative plastidial isoform might differ significantly from the cytosolic form, so that the anti-HPPD antibodies used in this study did not cross-react. The sensitivity of HPPD, like most non-haem-iron proteins, to H_2O_2 [5] renders its localization in the cytosol more likely than in the peroxisomes. H_2O_2 is produced continuously in peroxisomes during the β-oxidation of fatty acids [35] and by glycollate oxidase (EC 1.1.3.1) [36]. Thus, considering the sensitivity of HPPD to small amounts of H_2O_2 [5], and the very poor affinity of catalase for its substrate [37], it is difficult to understand how HPPD could operate in peroxisomes. Indeed, in mammals HPPD has been localized in the cytosol [38].

The existence and the subcellular localization of different isoforms of HPPD in plants remain to be clarified, and further work is still needed to address precisely this important point, and to characterize this enzyme activity biochemically.

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