Recombinant *p*-Hydroxyphenylpyruvate Dioxygenase of High Activity

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Inhibitors of *p*-hydroxyphenylpyruvate dioxygenase (HPPD) are bleaching compounds impairing the formation of colored carotenoids. This activity makes them promising candidates for herbicides. Detailed studies on enzyme-inhibitor complexes or on the binding niche of the enzyme have still to be performed. Enzyme preparation from plants is time-consuming and the yield is poor. This paper describes in relevant detail the preparation of recombinant enzyme from *Arabidopsis thaliana* with good yield and high specific activity.

Key words: Recombinant *p*-Hydroxyphenylpyruvate Dioxygenase, HPPD, Triketones

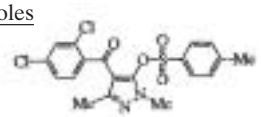
Introduction

p-Hydroxyphenylpyruvate dioxygenase (HPPD) catalyzes the conversion of hydroxyphenylpyruvate (HPP) to homogentisate (HGA). HPPD is a key enzyme in the biosynthesis pathway of plastoquinone and α-tocopherol. Plastoquinone is a hydrogen acceptor both in the photosynthetic electron transport system and in the biosynthesis pathway of desaturated carotenoids. Lack of plastoquinone due to the presence of HPPD-inhibitors will impair both processes (Pallett *et al.*, 1998). Concurrently with abolished carotenoid formation chlorophyll is degraded. HPPD-inhibitors do not act on the phytoene desaturase (Sandmann *et al.*, 1990) although the same symptoms due to phytoene desaturase-inhibitors are produced which interfere directly with the formation of functional carotenoids (Sandmann, 2002). Both inhibitor types are socalled "bleaching compounds". They can only produce a bleaching (and death of a cell) in growing meristemic parts of the leaf leaving intact the differentiated parts already green. Accordingly, bleaching herbicides are advantageously applied pre-emergent.

HPPD-inhibitors are relatively recent compounds and have been investigated since the 1990s. Many structures have been published (Hirai *et al.*, 2002), and some reached the commercial level as herbicides. Table I shows HPPD-herbicides belonging to three groups, namely pyrazoles, triketones and isoxazoles. All compounds include a benzoyl core, and at least two keto groups are required for inhibitory activity. A keto group in addition to that of the benzoyl may be formed by chemical conversion in the cell as is the case with isoxaflutole or pyrazolate (Viviani *et al.*, 1998; Hirai *et al.*, 2002).

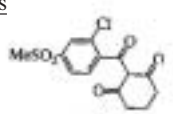
Table I. Examples for HPPD herbicides.

Pyrazoles

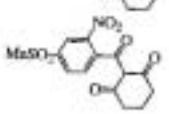


Pyrazolate

Triketones

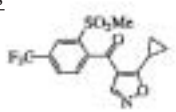


Sulcotrione



Mesotrione

Isoxazoles



Isoxaflutole

It should be noted that the dioxygenase is inhibited by certain natural triketones like usnic acid (Romagni *et al.*, 2000) or leptospermone (Hellyer, 1968; see also Meazza *et al.*, 2002). HPPD and its inhibition is not only a matter for the agrochemical industry but also for basic plant biochemistry. Several reports on properties of the isolated and (partly) purified plant dioxygenase have been pub-

lished (Secor, 1994; Barta and Böger, 1996; Viviani *et al*., 1998). The activity found of approx. 1–2 nmol mg^{-1} protein min^{-1} and the yield are rather low. Some X-ray studies and models are available using HPPD from *Pseudomonas fluorescens* (Serre *et al.*, 1999; Wu *et al.*, 2002; or Kakidani and Hirai, 2003). Only recently an excellent X-ray study on plant HPPD was published (Fritze *et al.*, 2004).

Further studies are required on inhibitor binding, and to elucidate details on the enzymatic mechanism. Garcia *et al.* (1999) used a cloning system which yielded an activity of 80 nmol min^{-1} mg $protein^{-1}$. This prompted us to modify their procedure and that of other authors with the objective to further increase the expressed activity of the recombinant HPPD by a reliable, time-saving and low-cost method for enzyme extraction and activity determination. This was achieved and the present paper describes in detail the method to make it usable for the investigator at the bench.

Materials and Methods

Cloning of Arabidopsis HPPD cDNA

HPPD coding sequence was amplified from total cDNA derived by RT-PCR of drought- stressed *Arabidopsis thaliana* leaf RNA. Specific primers were used for amplification and introduction of appropriate restriction sites (forward primer: 5′-cgg gat ccg cac caa aac gcc gc-3′, restriction site BamHI; reverse primer: 5′-gcg ccc ggg tca tcc cac taa ctg ttt ggc-3′, XmaI-site; restriction sites underlined) employing the Triple Master PCR system of Eppendorf (Hamburg, Germany; PCR-conditions: annealing temperature 59.7 °C, 20 cycles, 1 mM $MgCl_2$, proof reading Taq polymerase and buffers as recommended by Eppendorf).

The PCR-product was cloned into the pCR 2.1-Topo vector (Invitrogen, Carlsbad, CA, USA) and transferred to chemocompetent *Escherichia coli* DH5α for propagation (selection by ampicillin, 100 μg/ml). After extraction, purification and digestion of the HPPD-pCR 2.1-Topo vector, the BamHI/XmaI fragment was purified and subcloned in frame into the expression vector pQE31 (Qiagen, Hilden, Germany) and transferred to various *E. coli* strains [DH5α, M15(pRep4) or SG13009; the first strain is available from Invitrogen, the others from Qiagen].

Transformation of HPPD-pQE31 into different *Escherichia coli* strains was performed using a modification of the TSS-transformation of Chung *et al*. (1989). In brief, above *E. coli* cultures were grown to an optical density (OD) at 600 nm of 0.4; 1 ml thereof was transferred into an Eppendorf tube and centrifuged at full speed for 1 min. Plasmid DNA (0.5–2 μg of the HPPD-pQE31 per transformation) and the *E. coli* pellet were resuspended in filter-sterilized (0.2 μm) 100 μl TSS-solution (10% PEG 8000, 5% DMSO, 50 mM $MgCl_2$ in TB-medium; for TB-medium composition after Loewenstein-Jensen see http://www.sigmaaldrich.-com). After incubation on ice for 30 min, 0.8 ml TB-medium was added for phenotypic expression and incubation was continued for 60 min at 37 °C, shaking at 600 rpm in an Eppendorf tube shaker. *E. coli* were plated on LB agar containing appropriate antibiotics. Selection of HPPD-pQE31-positive *E. coli* was achieved by addition of ampicillin [100 μg/ml for DH5α, M15(pRep4), SG13009] and kanamycin [50 μg/ml for M15(pRep4), SG13009].

Selection of transformants containing an appropriately active HPPD-gene was performed by a combination of molecular biological colony screening (based on integration of the HPPD-cDNA into the pQE31-plasmid) and biochemically based screening on active HPPD-producing clones, showing a brown-reddish phenotype when grown in media supplemented with tyrosine (1 mM) due to the appearance of a polymerized oxidation product of homogentisic acid after cultivation (Denoya *et al*., 1994).

Cultivation of HPPD overexpressing E. coli

Cultivation was started with a 5-ml overnight culture inoculum in 0.5 l medium [LB, NZY, 2YT, TB, respectively; for culture media see Sambrook *et al.* (1989)] supplemented with appropriate antibiotics, namely ampicillin for HPPD-pQE31 containing DH5α strain (100 μg/ml), ampicillin plus kanamycin for strains M15(pRep4) or SG13009 containing HPPD-pQE31 (100 μg/ml and 50 μg/ml, respectively; for media composition see http://www.sigmaaldrich.com) and grown in 2-l flasks with baffles at 37 °C, 250 rpm until an OD at 600 nm of 0.6 to 0.8 was attained. In some cases, media were supplemented with tyrosine (up to 5 mM) and/or $FeSO_4$ (1 mM). Protein expression in pre-grown cultures was induced with 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG). After a standard induction period of 16 h at 22 °C, *E. coli* was harvested by centrifugation at 4 °C, 15 min,

4,200 × *g*. Protein expression pattern was analyzed by SDS-PAGE analysis for the overexpressed HPPD as an IPTG-inducible protein of ca. 50 kDa at various time courses (1 h up to 36 h induction) or various temperatures (22 °C, 30 °C, 37 °C).

Protein extraction

Preparation of crude protein extract was based on an adapted method of Sturner *et al.* (2000). Pellets of harvested *E. coli* cultures were resuspended in ice-cold extraction buffer [50 mM Tris-HCl (tris-(hydroxymethyl)aminomethane), pH 7.5, 1 mM dithiothreitol (DTT), 1 mg/ml lysozyme, 50 μg/ml DNase] at a ratio of 1/6 for (g fresh weight *E. coli*)/(ml extraction buffer). After an incubation period on ice for 15 min, *E. coli* cells were disrupted by sonification (in total 5 min sonification time, intervals of 30 s, settings: cycle 0.8, amplitude 80; ultrasonic laboratory device UP 50H; Dr. Hielscher, Tetlow, Germany). Warming of the samples due to ultrasonic treatment was avoided by keeping the cultures on an ice/NaCl mixture. After sonification, cell debris was removed by centrifugation at 4 °C, 20,000 × *g*, 30 min and the supernatant used for activity tests of HPPD. Total protein was determined according to Bradford (1976).

Determination of HPPD-activity by HPLC detection of homogentisate

Activity testing of crude HPPD-extracts was performed essentially according to Garcia *et al.* (1999). In brief, 100 mM Tris-HCl, pH 7.5, 50 mM Na-ascorbate, 200 μl 4-hydroxyphenyl-pyruvate, and 5–200 μg crude protein extract (depending on specific activity) were incubated for 15 min at 30 °C (unless otherwise noted), stopped by protein precipitation with 70 μl 20% (w/v) perchloric acid and subsequently cleared by centrifugation at 16,000 × *g*, 4 °C for 10 min. HPLC-separation of the reaction mixture (20 μl injection volume) was modified after Denoya *et al.* (1994) using an ABZ-plus column (Supelco, Bellefonte, PA, USA) at a flow rate of 1 ml/min and a gradient over 30 min of 95% buffer A (H_2O, 0.1% TFA), 5% buffer B (acetonitrile) for 0–5 min; 95% A, 5% B to 30% A, 70% B for 5–15 min; 30% A, 70% B to 0% A, 100% B for 15–20 min; 100% B for 20–25 min and 100%B to 95% A, 5% B for 25–30 min. The relative absorbance measured at 295 nm was correlated with amount of substance (nmol) of homogentisic acid by quantification of a standard curve obtained with commercially available homogentisic acid.

Results and Discussion

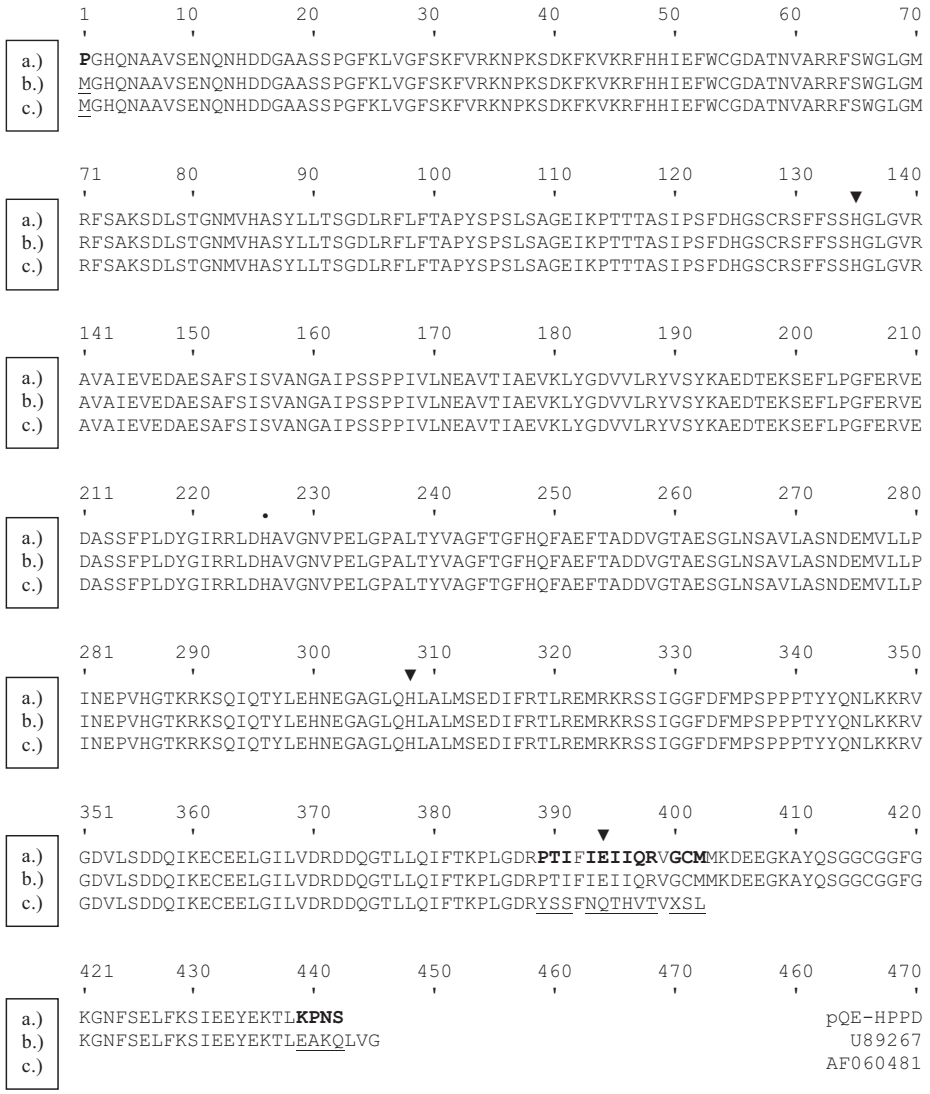
Cloning and sequencing

Sequence analysis and HPPD-activity assays revealed that the majority of (putative) HPPD-pQE31 harboring *E. coli* did not express functional HPPD, making a thorough screening necessary. The difficulty to screen each clone either genetically or by enzymatic assay can be circumvented due to the non-enzymatic oxidation of the product homogentisate, resulting in the formation of a red-brownish pigment. Based on visual survey of the browning of *E. coli* cultures and the subsequent exudation of the color into the surrounding medium (more prominent when the media were enriched in tyrosine), we were able to preclude non-functional HPPD-clones easily without sequencing all insert-positive ones. Hence, the combination of molecular analysis and biochemical screening is an efficient starting point for appropriate HPPD-cloning, allowing for a high throughput screening.

Sequence of the cloned HPPD coincided with published HPPD-sequences from *Arabidopsis thaliana* to a nearly perfect identity match except for minor differences in the C-terminal parts of the cDNA (Fig. 1). Published HPPD-sequences from different species include highly conserved regions at the C-termini, suggesting these domains have a function in the catalytic process (Garcia *et al.*, 1997). As we obtained very high specific activity with this clone (see below), apparently the amino acid exchanges observed in our case do not affect enzymatic turnover negatively.

Growth and extraction conditions

Variation in growth media composition can affect bacterial propagation and the yield of overexpressed active protein. In HPPD-overexpressing strains this was quantified measuring the absorbance of cultivation media at 450 nm (polymerized oxidation product of homogentisic acid, see above). Doing so, the *in vivo* estimation of different cultivation media for HPPD-production gave a rating (in ascending order) of NZY, LB, 2YT and – most suitable – TB culture medium (data not shown). More exact determination of specific HPPD-activity confirmed these findings and proved applicability of the method estimating



a.) Sequence pQE-HPPD
b.) Sequence U89267 (GenBank)
c.) Sequence AF060481 (GenBank)

▼ denotes the iron coordinating residues (see Fritze *et al*., 2004)
bold letters: amino acid exchanges of the pQE-HPPD in comparison to different GenBank sequences
italic letters: divergences of the GenBank sequences in comparison to the pQE-HPPD sequence

Amino acid exchanges in comparison of pQE-HPPD to U89267:
N-terminal: M1P due to introduction of restriction site for sub-cloning
C-terminal: E439K, A440P, K441N and Q442S and stop-codon after aa442 due to introduction of restriction site for sub-cloning (shortened sequence probably not relevant for enzymatic activity since the ordered structure ends with Helix11 at T447, see Fritze *et al*., 2004)

Fig. 1. Comparison of the amino acid sequence of recombinant HPPD-pQE31 (a) with published *Arabidopsis* HPPD-sequences from the GenBank (b, c).

activity by measuring the absorbance of the culture media. LB-grown *E. coli* DH5α had an extractable activity of 1.8 nmol homogentisate min^{-1} mg^{-1}, while 2YT-grown *E. coli* DH5α extract had an activity of 3.3 nmol homogentisate min^{-1} mg^{-1} protein. In summary, we found so-called rich or complex media (surplus of yeast extract, addition of glycerol as alternative carbon source and buffering of pH) superior to media with less complex composition.

Supplementation of media with $FeSO_4$ (to support adequate supply for the non-heme iron-cofactor of HPPD) or tyrosine (in order to circumvent tyrosine shortage due to branching towards HPPD) did not substantially upgrade expression and activity levels.

Extraction efficiency was comparable with cells broken up by either pressure or sonification (under the conditions as described in Materials and Methods), but the latter method was by far less time-consuming, hence possible protein degradation and oxidation were avoided.

The crude extract showed remarkable stability since storage at room temperature resulted in residual enzyme activities of approx. 60–80% after 16 h or 40–50% after 30 h. Nonetheless, enzyme activity in crude extracts was stable at −20 °C for a few weeks, thereafter, or after multiple thawing, loss in activity occurred and spontaneous formation of homogentisate was observed. Regeneration of the inactivated HPPD by incubation with ascorbic acid and/or Fe(II) salts as described by Linden (2000) was not successful, indicating that time-dependent inactivation was not caused by reversible loss or oxidation of the Fe-moiety. HPPD as a non-heme iron protein is very sensitive to H_2O_2, which may explain the slow but inevitable inactivation.

Improvement of HPPD-activity

One of our objectives was to establish a consistent, time-saving and straightforward method for measuring HPPD-activity *in vitro*.

In our hands, the photometric test on HPPD-activity based on the spectral shift resulting from keto-enol-tautomerism (modified after Lindstedt and Rundgren, 1982) did not produce reliable data; neither did the determination of HPPD-activity by oxygen-consumption [measurement according to Garcia *et al.* (1999)]. The latter method was hampered by high rates of non-specific oxygen-consumption, which could not be suppressed by NaN_3 or catalase. We assume that in both cases some non-specific side reactions occur due to the crude non-purified protein, superimposing HPPD-catalyzed reactions. We refrained from further purification steps (with concurrent activity losses) since our objective was to preserve an high-yielding HPPD-preparation, and adapted the activity test based on HPLC-separation of the substrate hydroxyphenylpyruvate and the product homogentisate. In brief: we performed the *extraction* modified after Sturner *et al.* (2000); *activity* test according to Garcia *et al.* (1999); and *HPLC separation* according to Denoya *et al.* (1994). The combination of these modified methods provided a sensitive and reliable tool for measurements of activity and inhibition (see Fig. 2).

An improvement of the assay conditions was achieved by variation of the pH value of the assay buffer. At pH 6.8, an activity of 1.7 nmol homogentisate min^{-1} mg^{-1} was measured, whilst the activity at pH 7.5 was 2.9 nmol homogentisate min^{-1} mg^{-1}. Neither a surplus of ascorbic acid nor the addition of $FeSO_4$ (1 mM) to the assay buffer improved enzymatic turnover.

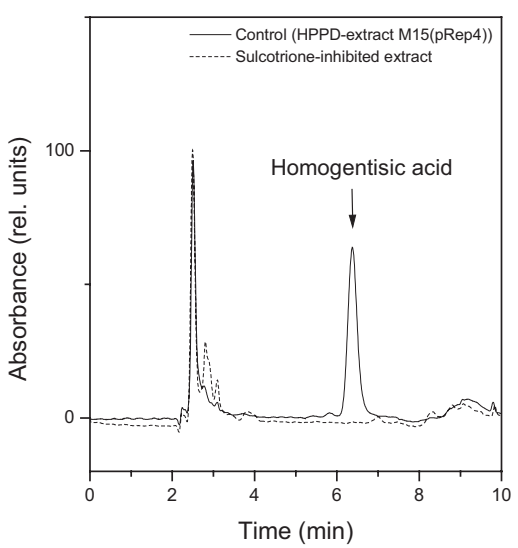


Fig. 2. HPLC-determination of homogentisic acid. Elution profile of HPPD-activity assays from non-inhibited preparations of HPPD-pQE31 M15(pRep4) extracts (straight line) and sulcotrione-inhibited preparations (dotted line).

Dependence of expression of HPPD-activity on different E. coli strains

As described earlier, the expression level strongly depends on the expression system used. Besides the tuning of cultivation conditions (see section above), the choice of appropriate expression vectors and bacterial strains defines the basis for an efficient overexpression system. In rare cases, combination of *E. coli* strain, expression vector and heterologous gene (and increasing enzyme protein) can trigger incompatibility, resulting in restricted expression levels or non-functional proteins, which sometimes are disposed in inclusion bodies. Since HPPD is relatively hydrophilic as judged from the amino acid sequence, the soluble nature of the protein prevents adverse effects as occurring with membranous proteins. As reported, specific activities of recombinant *Arabidopsis* HPPD differ in an order of magnitude. We used the pQE-vector for heterologous protein expression in combination with diverse *E. coli* strains [DH5α, M15(pRep4) and SG13009], and found activity ranges for the different strains varying to a large extent as well.

Strain DH5α, primarily used for the cloning steps and plasmid amplification (see Materials and Methods), was employed for expression studies, although it is not a typical strain for protein expression. Specific activity from this strain was only moderate [4 nmol homo-gentisate min^{-1} mg^{-1} protein (vector pQE31)] as compared to literature values with similar expression systems in DH5α [*e.g.* 45 nmol homogentisate min^{-1} mg^{-1} with vector pKK233–2; Sturner *et al.* (2000)]. The discrepancy in specific activity could arise from the vector-host combinations or is due to differences in determination of activity. [For example, activity assays by Sturner *et al.* (2000) were performed with desalted extracts and based on a radioactive assay.]

The other two *E. coli* strains tested – M15(pRep4) and SG13009 – which are typical protein overexpressing strains, yielded by far higher activities of recombinant HPPD. While the extractable specific activity with SG13009 was in the range of 110–200 nmol homogentisate min^{-1} mg^{-1}, extracts from M15(pRep4) exhibited an activity of 170–260 nmol homogentisate min^{-1} mg^{-1} which was not reported before. As mentioned above, the particular combination of overexpressed gene and *E. coli* strain seems to determine extractable good HPPD-activity. For instance, overexpression of *Arabidopsis* HPPD in the strain HMS174(DE3) yielded 13 nmol homogentisate min^{-1} mg^{-1} protein (Linden, 2000). Overexpression in strain BL21(DE3) yielded 30 nmol homogentisate min^{-1} mg^{-1} (Fritze *et al.*, 2004), and JM 105 produced an activity of 80 nmol homogentisate min^{-1} mg^{-1} (Garcia *et al.*, 1999).

Inhibition of the recombinant HPPD by sulcotrione

The herbicide sulcotrione, 2-(2-chloro-4-methanesulfonylbenzoyl)-1,3-cyclohexanedione, is a specific inhibitor of HPPD, excerting its effect on the purified enzyme at nanomolar concentrations. We found the half-maximum inhibition (I_{50}) at 0.7 μM for sulcotrione without preincubation (tested with extracts from DH5α, Table II). Literature values for recombinant *Arabidopsis* HPPD tend to be somewhat lower (*e.g.* I_{50} 0.1 μM; Sturner *et al.*, 2000). Possibly, the slightly higher I_{50} values we obtained are due to the crude protein preparations we used without purification steps.

Preincubation of the enzyme with sulcotrione for 15 min, however, significantly enhanced the inhibition (tested with extracts from SG13009, Fig. 3). Direct addition of sulcotrione (1 μM) to the assay inhibited to about 40%, whereas a 15-min incubation with 1 μM sulcotrione prior to measurement diminished the activity to about 10% of the non-inhibited control. A 15-min preincubation of HPPD with the inhibitor sulcotrione resulted in an I_{50} value of 0.3 μM [tested with extracts from M15(pRep4); Fig. 4], which again is markedly lower than the I_{50} without preincubation (0.7 μM). Since sulcotrione is a tight-binding type inhibitor [see Viviani *et al.* (1998)], this finding with overexpressed HPPD confirms the enzymatic characteristics previously found with the native enzyme.

Table II. Inhibition of recombinant *Arabidopsis* HPPD by sulcotrione. The activity of non-inhibited DH5α extracts was 4 nmol homogentisate min^{-1} mg^{-1}, inhibition by sulcotrione was assayed without preincubation.

Sulcotrione [μM]	Activity (% of control)	SD
0	100	
0.5	58.0	± 15.1
1.0	21.3	± 6.5
1.5	2.8	± 1.1

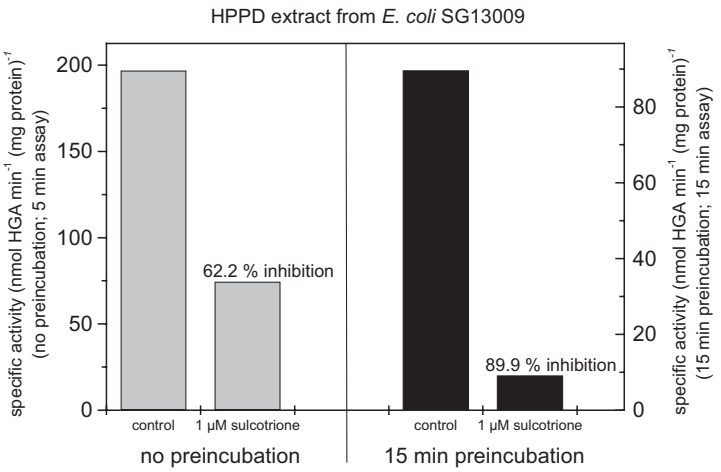


Fig. 3. Inhibition of recombinant *Arabidopsis* HPPD by sulcotrione with and without preincubation. Extracts from HPPD-pQE31 SG13009 were either co-incubated with sulcotrione (grey columns) or preincubated with sulcotrione (prior to the activity assay for 15 min, black columns) and evaluated for inhibition as compared to the corresponding controls. Control values, gained by addition of the sulcotrione solvent acetone solely, varied to some extend (remark the different ordinates) due to different exposure time to the solvent.

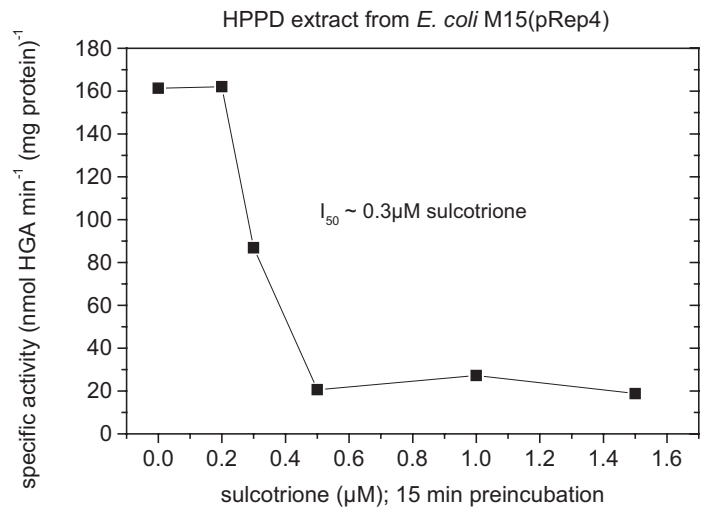


Fig. 4: Inhibition of recombinant *Arabidopsis* HPPD by sulcotrione with preincubation. Extracts of HPPD-pQE31 M15(pRep4) were tested for inhibition by sulcotrione with 15 min preincubation prior to the assay.

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