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A novel amperometric biosensor for ß-triketone herbicides based on hydroxyphenylpyruvate dioxygenase inhibition: A case study for sulcotrione

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ABSTRACT

An amperometric biosensor was designed for the determination of sulcotrione, a β-triketone herbicide, based on inhibition of hydroxyphenylpyruvate dioxygenase (HPPD), an enzyme allowing the oxidation of hydroxyphenylpyruvate (HPP) in homogentisic acid (HGA). HPPD was produced by cloning the *hppd* gene from *Arabidopsis thaliana* in *E. coli*, followed by overexpression and purification by nickel-histidine affinity. The electrochemical detection of HPPD activity was based on the electrochemical oxidation of HGA at +0.1 V vs. Ag/AgCl, using a poly(3,4-ethylenedioxythiophene) polystyrene sulfonate-modified screen-printed electrode. Assays were performed at 25 °C in 0.1 M phosphate buffer pH 8 containing 0.1 M KCl. The purified HPPD was shown to display a maximum velocity of 0.51 μM_{HGA} min^{-1}, and an apparent K_M of 22.6 μM for HPP. HPPD inhibition assays in presence of sulcotrione confirmed a competitive inhibition of HPPD, the calculated inhibition constant K_I was $1.11.10^{-8}$ M. The dynamic range for sulcotrione extended from 5.10^{-10} M to 5.10^{-6} M and the limit of detection (LOD), estimated as the concentration inducing 20% of inhibition, was $1.4.10^{-10}$ M.

1. Introduction

4-hydroxyphenylpyruvate dioxygenase (HPPD; EC 1.13.11.27) belongs to α-keto-acid-dependent, non-hemic, Fe^{2+}-dependent enzymes [1–3]. It has been isolated from various organisms such as bacteria [1,4–7], mammals [3,8–11], and plants [10,12–16]. These studies have shown that HPPD can be present in two active forms, as a homodimer [10,12,15–17], or a homotetramer, mainly found in bacteria [1,5,18]. Both active forms have approximately subunits of 40–48 kDa.

HPPD is involved in tyrosine catabolism [19,20], which is essential for most aerobic living organisms. Tyrosine aminotransferase (TAT; EC 2.6.1.5) is the first enzyme of this pathway and converts L-tyrosine into hydroxyphenylpyruvic acid (HPP) by transamination [21] (see Eq. (1)). HPPD catalyzes the conversion of hydroxylphenylpyruvate (HPP) into homogentisic acid (HGA) [22,23] (see Eq. (2)), this complex reaction involves oxidative decarboxylation of HPP side chain, hydroxylation of the aromatic ring and migration an alkyl group [9,16,24]. HGA functions vary depending on the organism in question. In mammals, HGA allows producing intermediate products yielding to energy-rich products, acetoacetate and fumarate [11]. In photosynthetic organisms, HGA is a key precursor of α-tocopherol and plastoquinone, both essential cofactors in photosynthesis ([3,25]). However, other studies have shown that HGA can be involved in another catabolic pathway, similar to that of mammalian [26].

Tyr + 2-oxoglutarate → **HPP** + L-glutamate (1)

HPP + O_2 → **HGA** + CO_2 (2)

Plant HPPD is the biochemical target of a range of synthetic β-triketone herbicides that are currently commercially available such as sulcotrione, mesotrione and tembotrione [3,25,27–29]. These herbicides are chemically derived from the natural phytotoxin leptospermone produced by the Californian bottlebrush plant *Callistemon citrinus* [3,30,31]. Synthetic β-triketones are commonly used as post- and pre-emergent selective herbicides for protecting maize crops against grass weeds, they have efficiently replaced atrazine, banned in several European countries since 2003 [32].

Sulcotrione (2-[2-chloro-4-(methylsulfonyl)benzoyl]-1,3-cyclohexanedione) (Fig. 1) was introduced on the market in 1993 by Bayer CropScience SA under the trade name Mikado®. Sulcotrione is generally used at a rate of 450 g ha^{-1}, it is described as short persistent, with a half-life in field varying between 16 and 122 days, and relatively mobile in water, with an adsorption coefficient varying from 0.66 to 2.82 [33,34]. Sulcotrione biotic and abiotic

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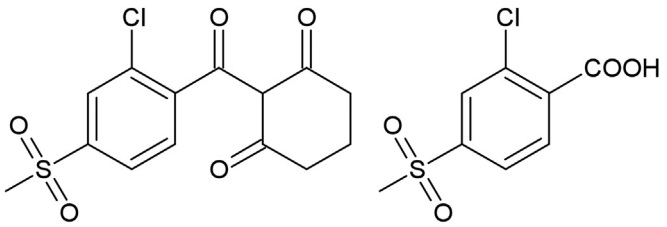

Fig. 1. Sulcotrione and its main degradation product, CMBA.

degradation generates mainly CMBA (2-chloro-4-methylsulfonylbenzoïc acid) (Fig. 1) [35–38], which exhibits a higher toxicity than the parent compound in the Microtox bioassay [39].

Inhibition of the HPPD enzyme by β-triketone herbicides leads to the decrease of HGA level, inducing a depletion of plastoquinone, which is an essential cofactor of phytoene-desaturase, an enzyme involved in catotenoids biosynthesis. The induced depletion of catotenoids leads to a decrease of chlorophyll protection, resulting in foliage bleaching and plant death [3,25,40].

The most common conventional method currently available for the determination of sulcotrione is high-performance liquid chromatography (HPLC) combined with different detection techniques such as UV [34,41–43], tandem mass spectrometry [44], or mass spectrometry [41]. Despite these techniques can reach low limits of detection (LOD), in the nanomolar range [44], they are mainly devoted to laboratory analysis, they involve very expensive apparatus and are not adapted to in situ measurements.

As a consequence and in the context of the REACH European regulation (2006), which makes industry responsible for assessing and managing the risks posed by chemicals, industrials are particularly interested in alternative methods for the assessment of hazardous substances. These methods should be ideally fast, sensitive, reliable, easy to handle, and they should allow high frequency measurements.

Up to now, only one alternative tool was developed for the detection of the sulcotrione, based on a whole-cell colorimetric bioassay involving a recombinant *E. coli* strain expressing *Arabidopsis thaliana* HPPD. The bioassay principle relies on the capacity of the recombinant clone to produce through tyrosine catabolism a soluble melanin-like pigment, detectable at 405 nm. In presence of an HPPD inhibitor, a decrease in the pigment production is observed which is proportional to inhibitor concentration. Using such a system, we previously reported the detection of sulcotrione with a LOD value of 0.038 μM [45]. Another advantage of this bioassay consisted in the possibility of carrying out simultaneously multiple analyses in less than 12 h.

Based on these promising results, and taking into account the possibility of extracting and purifying the enzyme, the development of a rapid detection tool based on biosensor technology was considered. Biosensors are described as greatly attractive alternative detection methods, due to their inherent characteristics including high sensitivity, real time measurement and possibility of in situ monitoring. Up to date, the great majority of enzymatic sensors described in the literature are devoted to the detection of acetylcholinesterase inhibitors, mainly organophosphorus and carbamate insecticides [46,47]. The objective of this work was to evaluate the possibility of designing for the first time an amperometric enzymatic biosensor based on the sulcotrione ability to specifically inhibit HPPD.

2. Material and methods

2.1. Optimization of HPPD expression

Cloning of the *hppd* gene from *Arabidopsis thaliana* into the expression vector pET 16b was described in a recent work [45], as well as optimal parameters of the HPPD overexpression in *E. coli* BL21 (DE3). In order to enhance the HPPD expression level, several strains of *E. coli* were tried out: *E. coli* BL21 (DE3) pLysS and *E. coli* BL21 (DE3) Star pLysS. These two strains carry a chloramphenicol-resistant plasmid with a P15A replicon that encodes T7 lysozyme, a natural inhibitor of T7 RNA polymerase. Basal expression of T7 RNA polymerase is thus suppressed/reduced before and during induction period. mRNA stability may eventually be enhanced by the use of *E. coli* BL21 Star pLysS strain which has an additional mutation in the RNaseE gene (*rne131*), involved in mRNA degradation.

E. coli cells harboring the pET16b–HPPD plasmid, and named *E. coli* HPPD, were grown at 30 °C in a fresh LB supplemented with antibiotics. Ampicillin was used at a final concentration of 100 μg mL^{-1} for *E. coli* BL21 (DE3), while ampicillin and chloramphenicol, were used for both *E. coli* BL21 pLysS and *E. coli* BL21 Star pLysS, at 100 μg mL^{-1} and 3 μg mL^{-1}, respectively. After an overnight culture, the recombinant strains were transferred in fresh LB media, supplemented by appropriate antibiotics, and grown up to an optical density of 0.4 at 600 nm (OD_{600}) (spectrophotometer UV-1800-Shimadzu, Kyoto, Japan). Then, protein expression was induced using isopropyl-β-D-thiogalacto-pyranoside (IPTG-Biosolve) under several experimental conditions. Three final concentrations of IPTG (0.1, 0.5 or 1 mM), three incubation temperatures (4 °C, 20 °C or 28 °C), and three induction times (3, 24 or 48 h) were assessed during incubation in an orbital agitator at 80 rpm.

2.2. Monitoring of HPPD activity using a colorimetric free-whole-cell assay

The colorimetric free-whole-cell assay based on the tyrosine degradation pathway was used as previously described [45], in order to ascertain HPPD expression and activity. The different induced cultures were directly used and mixed with an equal volume of L-tyrosine (Tyr) at 1.2 mg mL^{-1} (Sigma, Saint-Quentin Fallavier, France). Cell suspensions were incubated at 37 °C for 24 h, biomass variation was then evaluated for each sample by measuring OD_{600}. After a 10 min centrifugation at 8000 rpm, the supernatant was removed and optical density of the soluble brown pigment formed in the culture medium was measured at 400 nm (OD_{400}) (UV-1800-Shimadzu, Kyoto, Japan).

2.3. Extraction and purification of HPPD

Total crude cell extracts were prepared from 2.88 mg of the induced *E. coli* HPPD resuspended in 1X loading buffer (6 g L^{-1} Tris–HCl, pH 6.8, 4% SDS, 0.02% bromophenol blue and 3% dithiothreitol). The bacterial-cell pellet was disrupted by heat shock (95 °C, 10 min) and then cleared by centrifugation. For protein extraction, the induced *E. coli* HPPD cells were harvested and stored at −20 °C before a chemical disruption with Bacterial Protein Extraction Reagent (B-PER) (Thermo Scientific). Thawed pellets were then resuspended in B-PER containing a protease inhibitor cocktail (Pierce, Rockford, USA), 1.10^{-2} mg mL^{-1} DNAse (Sigma, Saint-Quentin Fallavier, France), 0.02 M $MgCl_2$ (Sigma, Saint-Quentin Fallavier, France) and 10 mM ascorbic acid. The mixture was incubated 20 min at room temperature. Cell debris were removed by centrifugation (10 min, 5000 rpm, 4 °C) and supernatant was stored at 4 °C before purification. Crude cells or

protein extracts were analyzed by SDS-PAGE for protein expression pattern. Purification was performed on a HisPur Ni-NTA resin (Thermo Scientific) after dilution (1:1) of the clarified supernatant with binding buffer (20 mM sodium phosphate, 300 mM NaCl and 250 mM imidazole, pH 7.4). Purification procedure was then applied as suggested by the manufacturer. Protein expression pattern and protein purity were investigated by SDS-PAGE. HPPD fractions were stored at −20 °C in 20% glycerol (Sigma). Total protein concentrations were measured using the Bradford assay [48], with bovine serum albumin as standard.

2.4. Electrochemical studies

Cyclic voltammetry experiments were performed for studying the electrochemical properties of HPP and HGA, which are respectively the HPPD substrate and product. Both compounds were used at a concentration of 5 mM in 0.1 M phosphate buffer (PB) (0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4) pH 7.6 containing 0.1 M KCl. Cyclic voltammetry measurements were carried out at room temperature using a conventional three-electrode system, unmodified or modified with poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS). Potential scanning was performed between −0.7 V and 0.7 V vs. Ag/AgCl at a scan rate of 50 mV s^{-1} and a step potential of 5 mV s^{-1}. Amperometric measurements were performed in a reaction cell (final volume 1.5 mL) under gentle stirring. The applied potential was scanned between −0.45 V and 0.25 V vs. Ag/AgCl (step 0.05 V). After stabilization of the current baseline, HGA and HPP were injected at a final concentration of 250 μM. All the experiments were carried out at room temperature.

2.5. Studies of HPPD activity

HPPD optimum activity was first studied at different pH ranging from 5 to 9, using various buffers (0.1 M HEPES, MOPS or PB containing 0.1 M KCl). The KCl concentration in buffer was then varied between 0.05 M and 0.2 M. The optimum temperature was studied using buffer thermostated at temperatures ranging from 35 to 45 °C. All the assays were performed by incubating the free enzyme with its substrate for 5 min in a 1.5 mL microtube under stirring at room temperature before injection in the reaction cell. In parallel, HPP was studied in the same conditions without enzymes. Calibration curves of HPP and HGA were realized for concentrations ranging from 0.001 mM to 5 mM. To determine the enzyme stability in liquid medium, HPPD was stored in the elution buffer at 4 °C during 24 h. Electrochemical assays were performed with 250 μM HPP before and after 24 h of storage at 4 °C. All the assays were realized in triplicate.

2.6. Assay and determination of kinetic parameters

The activity of purified HPPD was studied using HPP concentrations ranging from 0.0025 mM to 0.5 mM. The apparent Michaelis constant (K_M) and the maximum velocity (V_{max}) were estimated by linear fitting of the Lineweaver–Burk double-reciprocal plots using Origin Pro 8.6 (OriginLab Corporation, USA).

2.7. Biosensor determination of sulcotrione

Electrochemical behavior of sulcotrione at 0.2 mg L^{-1} was characterized by cyclic voltammetry in the same conditions as described above. Preliminary HPPD inhibition assays were performed by incubating the enzyme during time periods ranging from 2 to 10 min in presence of sulcotrione at 1.10^{-8} M and 1.10^{-6} M. The inhibition constant K_I was then determined by measuring apparent K_M using 3 HPP concentrations and 3 different concentrations of sulcotrione: 1.10^{-7}, 1.10^{-8} and 1.10^{-9} M. For biosensors assays, the procedure was similar as described above except that sulcotrione, was added at concentrations ranging from 1.10^{-18} to 1.10^{-1} M and incubated during 5 min before addition of 0.1 mM HPP substrate.

3. Results and discussion

3.1. HPPD expression and purification

In a previous work, HPPD overexpression was performed in *E. coli* (DE3) BL21 strain, it was demonstrated that HPPD level was higher in the soluble fraction following induction with 1 mM IPTG in LB medium at 4 °C during 48 h [45]. The lack of stability of the clone was the main issue of the use of this strain. To improve HPPD overexpression, the recombinant plasmid pET16b–HPPD was expressed in different host bacterial strains, including *E. coli* BL21 pLysS and *E. coli* BL21 Star pLysS, and experimental conditions such as induction time, temperature and IPTG concentration were optimized. After centrifugation, cells obtained under the different conditions were chemically lyzed and the soluble fraction was analyzed by SDS-PAGE (Tables 1 and 2).

No band was visible upon induction at 4 °C (Table 1). For both strains, a substantive expression level was observed upon induction at 28 °C, starting from 3 h to 24 h, characterized by a thick band at 48 kDa. Using longer induction times, this band was less visible in the soluble fraction (data not shown). The same phenomenon was observed at 20 °C with *E. coli* BL21 pLysS and *E. coli* BL21 Star pLysS. As shown in Table 2, the best expression level was obtained for both strains using an IPTG concentration of 0.1 mM, while *E. coli* BL21 required a 10-fold higher concentration of inductor (1 mM).

E. coli BL21 pLysS strain was selected for further studies due to its rapidity in reaching exponential phase: only 3 h of incubation were needed to reach an OD_{600} of 0.6, while 5 h were required for *E. coli* BL21 Star pLysS strain. Moreover, for analogous induction conditions and equal biomass, the use of BL21 pLysS strain allowed obtaining a stronger brownish pigmentation.

The purification of HPPD enzyme was facilitated by the use of pET16b vector which carries a 6-His tag, allowing its binding on immobilized metal affinity chromatographic column. The analysis of the purified fraction by SDS-PAGE electrophoresis showed a unique band at 48 kDa, corresponding to the molecular weight of *At*HPPD (Fig. 2).

3.2. Electrochemical studies and determination of the working potential

In a first step, the electrochemical behavior of the HPPD substrate (HPP) and product (HGA) at 5 mM was first studied by cyclic

Table 1
Temperature influence on HPPD overexpression level within 3 different *E. coli* strains. (+) Refers to the expression level, when a thick stained band on SDS-PAGE was observed at 48 kDa. (−) Indicates an absence of HPPD band.

	E. coli strains								
	BL21			BL21 pLysS			BL21Star pLysS		
Temperature	4 °C	20 °C	28 °C	4 °C	20 °C	28 °C	4 °C	20 °C	28 °C
Induction time (h)									
3	−	−	−	−	+	+	−	+	+
24	+	−	−	−	++	+++	−	++	+++
48	++	−	−	−	++	++	−	+	++

Table 2
Effect of IPTG concentration on HPPD overexpression level within 3 different *E. coli* strains. (+) Refers to the expression level, when a thick stained band on SDS-PAGE was observed at 48 kDa. (−) Indicates an absence of HPPD band.

	E. coli strains		
IPTG concentration (mM)	BL21	BL21 pLysS	BL21Star pLysS
0.1	+	++	++
0.5	+	+	+
1	++	+	−

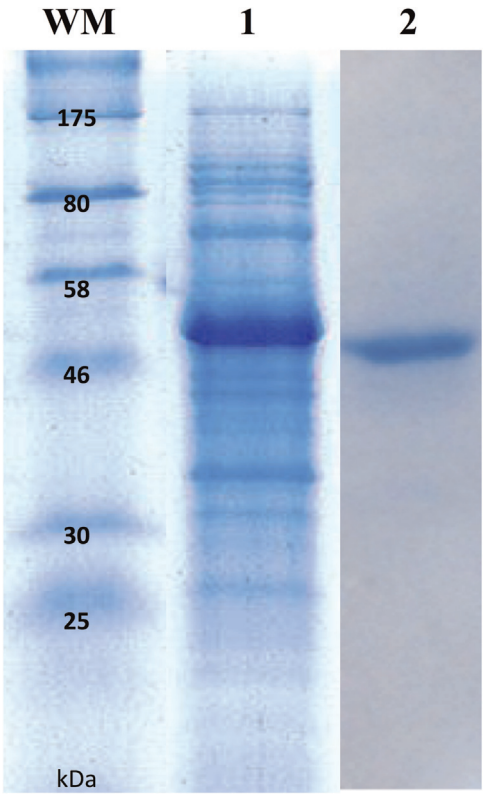

Fig. 2. SDS-PAGE profile of the recombinant *E. coli* HPPD strain before (1) and after IMAC purification (2) from a culture induced at 4 °C. WM: Molecular Weight Markers.

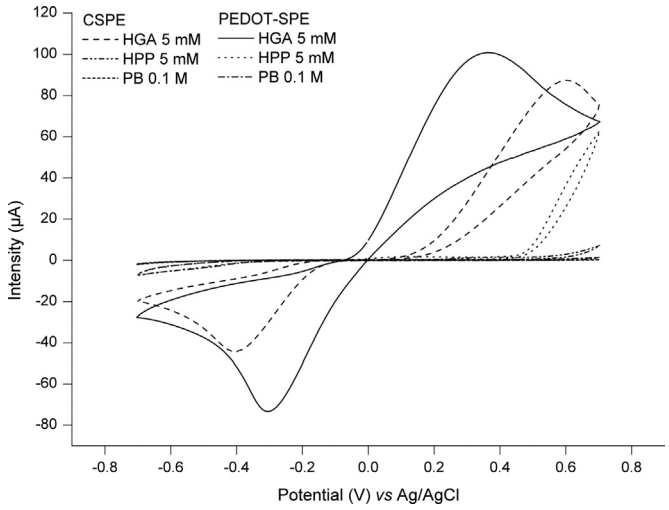

Fig. 3. Cyclic voltammograms obtained on CSPE and PEDOT–SPE for 5 mM of HPP and HGA in reaction buffer at pH7.6 and 25 °C.

voltammetry using Carbon Screen-Printed Electrodes (CSPE) modified or not with PEDOT:PSS. As shown in the voltammogram (Fig. 3), neither reduction nor oxidation of HPP could be observed in the tested potential range, whatever the electrode used. Previous works described PEDOT:PSS mediator as a very efficient electrical conductor [49] with a great stability, it was used to develop several biosensors such as an acetylcholinesterase-based sensor for insecticides monitoring [46]. The results confirmed this observation since the higher current intensities were observed in presence of PEDOT:PSS. The oxidation peak of HGA was observed starting at +0.60 V and +0.35 V vs. Ag/AgCl using respectively CSPE and PEDOT–CSPE. HGA reduction peaks were also observed at −0.40 V and −0.30 V vs. Ag/AgCl for CSPE and PEDOT-modified CSPE, respectively. These results demonstrate that PEDOT:PSS plays the role of electronic mediator for HGA detection, as it allows a dramatic lowering of both oxidation and reduction potentials. The incorporation of PEDOT:PSS in CSPE is thus likely to reduce potential interference due to oxidation/reduction of undesirable compounds.

In a second step, the optimal working potential was determined in amperometric conditions using PEDOT:PSS-modified CSPE. The oxidation or reduction current of 250 µM HPP or HGA was measured at fixed potentials ranging from −0.45 to +0.2 V vs. Ag/AgCl (data not shown). As previously described, the reduction of the substrate HPP was observed at potentials below −0.3 V vs. Ag/AgCl. A slight HPP oxidation current was observed at potentials ranging from 0 V to +0.2 V vs. Ag/AgCl. The current sensitivity calculated at +0.1 V was 0.16 mA M^{-1}. By opposition, a huge oxidative current was observed in presence of the product HGA at potentials ranging from −0.05 to +0.2 V vs. Ag/AgCl. A working potential of +0.1 V vs. Ag/AgCl was selected for the further experiments, resulting from the best compromise between the HGA oxidation response and the unspecific response due to HPP oxidation. At this potential, the calculated sensitivity to HGA was 5.6 mA M^{-1}.

3.3. Optimization of operational conditions

Numerous parameters can greatly affect the enzyme activity, one of the most important is pH that may lead to protein denaturation or contribute to the combination between the enzyme and its substrate [50]. As described in literature, previous works on HPPD activity have been performed using various buffers such as Tris–HCl [31], HEPES [15], MOPS [10] or PBS [25], with pH values varying from 7 [6] to 7.5 [51]. In these reports, HPPD activity was generally measured in stopped mode using HPLC analysis of the enzymatic product HGA [3,5,7,31,51,52]. As this work presents for the first time an electrochemical method for measuring HPPD activity, critical parameters such as pH and buffer composition were firstly investigated. The optimal pH was evaluated in a pH range from 5 to 9 using either 0.1 M HEPES, MOPS or PB solutions containing 0.1 M KCl. The use of Tris–HCl was avoided, as HPP was shown to be unstable in such buffer. The results presented in Fig. 4A show that the effect of pH on HPPD activity closely depends on the buffer used. It appears clearly that MOPS is not a convenient buffer for HPPD, even if a maximum activity was observed between pH 8 and 9. Using HEPES buffer, the current intensity increased progressively with pH until pH 7, and then suddenly increase until reaching a maximal value at pH 8. A similar behavior was observed using phosphate buffer, except that the increase of activity was observed starting at pH 6. The current intensity measured at pH 8 was globally 25% higher using PB than using HEPES, the current sensitivities being respectively of 3.01 and 4.05 mA M^{-1}. HPPD activity was then measured in PB at different concentrations, containing various KCl concentrations, ranging from 0.05 to 0.2 M. Previous studies have reported that these two

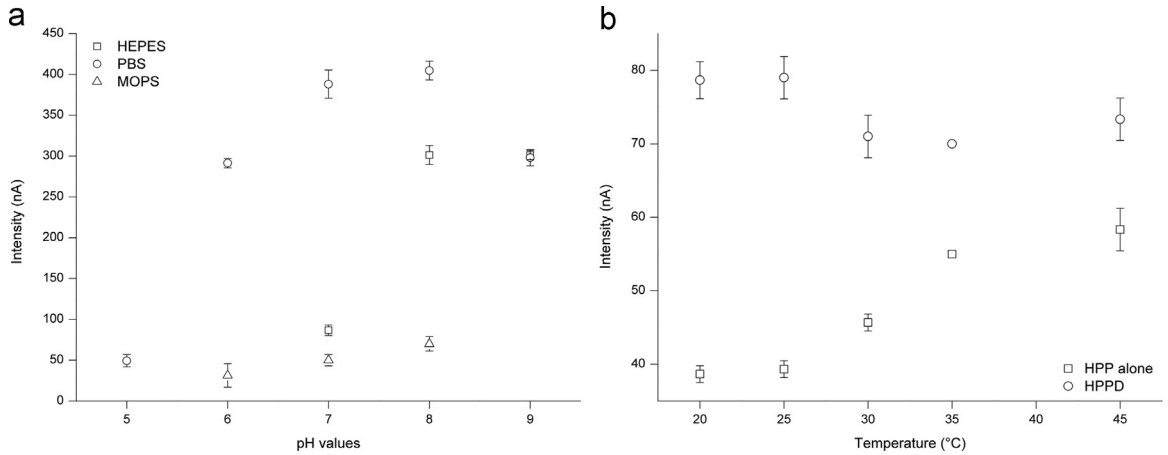

Fig. 4. Effect of buffer, pH (A) and temperature (B) on the response of HPPD biosensor.

Table 3
The apparent Michaelis constant, K_M, of HPPD from different organisms obtained by various analytical methods.

Analytical method	Organisms	K_M (μM)	References
Oxygen electrode	*S. avertimilis*	27	[6]
	A. thaliana	6.9	[15]
	D. carota	7.5	[16]
	H. sapiens	80	[57]
Radiometric assay	*A. thaliana*	11	[10]
	R. norvegicus	13	[10]
HPLC	*D. carota*	2.6	[28]
Electrochemical biosensor	***A. thaliana***	**22.6**	This study

factors could affect the enzymatic response, leading to some important fluctuations in the amperometric responses or limiting the enzyme activity [53–55]. The higher responses were reached using 0.1 M PBS containing 0.1 M KCl. In this case, the sensor sensitivity to HGA was calculated to be 1.36 mA M^{-1}. 0.1 M phosphate buffer pH 8 containing 0.1 M KCl was therefore selected for further experiments.

Temperature is another major parameter to take into consideration as enzymes and more generally proteins activity and denaturation are highly temperature-dependent phenomena. Enzyme activity generally increases up to an optimum temperature and then decreases to weaker activity at high temperatures. For determination of the optimum temperature, HPPD electrochemical assays were performed at different temperatures ranging from 20 °C to 45 °C. In parallel, HPP oxidation was also studied in the same range of temperature, using the same concentration as in HPPD assays. It was shown that HPPD displays a maximum activity at temperatures between 20 °C and 25 °C (Fig. 4B). At these temperatures, a weak HPP oxidation was observed, which was greatly enhanced when temperature was increased up to 45 °C. Previous studies have already reported such instability of HPP, which is likely due to spontaneous hydrolysis into 4-hydroxybenzaldehyde and 4 hydroxyphenyl acetic acid [56]. According to these results, it was decided to perform the amperometric measurements at room temperature, i.e. between 20 and 25 °C.

3.4. Calibration of PEDOT:PSS-modified CSPE

Calibration curves were first drawn using either HPP or HGA at concentrations ranging from 0 to 10^{-3} M. As expected, HGA calibration curve showed current responses significantly higher than those obtained with HPP. The linear regression equations were $y=26.9x \pm 0.9$ mA M^{-1} ($r^2=0.99$) and $y=0.8x \pm 0.03$ mA M^{-1} ($r^2=0.989$) for HGA and HPP, respectively. The sensitivity of PEDOT:PSS-modified CSPE to HGA was thus 34-fold higher than the sensitivity to HPP.

3.5. Electrochemical determination of HPPD kinetic parameters

Kinetic parameters of free HPPD were evaluated by electrochemical measurements using HPP concentrations ranging from 0.0025 mM to 0.5 mM. Kinetic constants values were estimated for the purified *At*HPPD in solution using the double reciprocal (1/v versus 1/[S]) Lineweaver–Burk plot. The resulting linear representation obeyed the equation $y=3.286.10^{-7}$ x$+1.4.10^{-3}$. The intercept on the $1/v$ axis allowed determining the maximum velocity $V_{max}=0.51$ μM_{HGA} min^{-1}, while the apparent Michaelis constant $K_M=22.6$ μM was calculated using the intercept on 1/[S] axis. This K_M value is in accordance with the data previously published in literature (Table 3), which reported that the K_M values of HPPD from different organisms were in the micromolar range. Even if *At*HPPD presented a great affinity for its substrate, the K_M value obtained in this work was 2 to 3-fold higher than the K_M value obtained with other analytic methods. This slight difference might be due to the fact that these studies involved partially purified or crude extracts, which allowed preserving the redox conditions necessary for optimal enzyme activity.

3.6. Biosensor determination of sulcotrione

Cyclic voltammetry was first used to study the electrochemical behavior of the β-triketone sulcotrione on the PEDOT:PSS-modified electrode. It was shown that this inhibitor did not exhibit any electrochemical signal at potentials ranging from −0.7 to +0.7 V vs. Ag/AgCl. Its main degradation product, CMBA, was also examined in CV and displayed a similar electrochemical behavior.

HPPD inhibition assays were performed by incubating the enzyme during time periods ranging from 2 to 10 min, in presence of sulcotrione at 1.10^{-8} M and 1.10^{-6} M. After addition of the substrate, the mixture was injected in the reaction cell and the residual current response was compared to the response in absence of inhibitor. The results showed that sulcotrione behaves as time-independent reversible inhibitor (data not shown). Similar results were previously described for natural β-triketones [31], and for the synthetic β-triketone NTBC [58,59]. However it is the first time that such behavior is observed using a purified HPPD and a synthetic β-triketone, namely sulcotrione. It must be stressed that these results were not really expected as synthetic β-triketones have been previously described as tight-binding molecules, mainly due to the presence of electron-withdrawing groups, such as $-SO_2CH_3$ in sulcotrione [3,31,59]. Inhibition kinetic analysis,

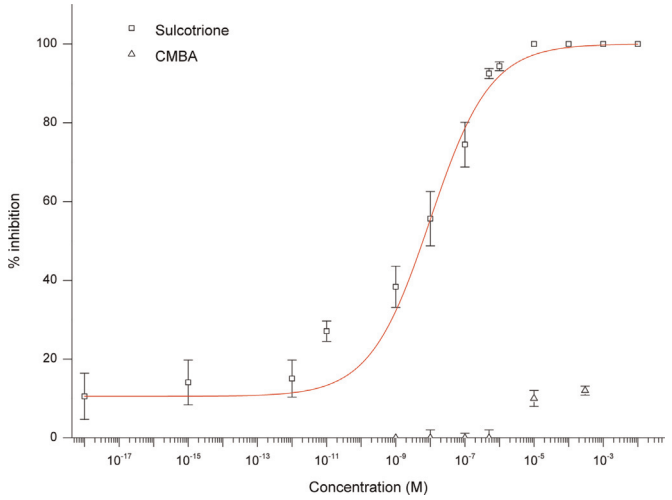

Fig. 5. Effect of sulcotrione and CMBA concentrations on the response of the biosensor based on free HPPD.

performed with 3 HPP concentrations and sulcotrione concentrations ranging from 0 to 1.10^{-7} M, showed that the apparent K_M increased with sulcotrione concentration. This behavior is consistent with the data present in the literature, describing sulcotrione as a competitive inhibitor of HPPD. Re-plotting the apparent K_M against inhibitor concentration allowed calculating an inhibition constant K_I of $1.11.10^{-8}$ M. This value was 2000-fold lower than the K_M obtained for HPP towards HPPD (26 μM), showing a very high affinity of sulcotrione for *At*HPPD. An inhibition curve was thus drawn using sulcotrione concentrations ranging from 1.10^{-25} M to 2.10^{-2} M, resulting in a sigmoidal plot (Fig. 5) obeying the following equation (Origin Pro 8.6) (Eq. (3)):

$$y = 10.53 + (100 - 10.53)/(1 + (9.8.10^{-9}/x)^{0.499}) \quad (3)$$

As shown in Fig. 5, the dynamic range for sulcotrione detection extended from 5.10^{-10} M to 5.10^{-6} M. CMBA, the main sulcotrione metabolite, was shown to be a very poor inhibitor of HPPD, a 1.10^{-5} M concentration inducing only 10% of inhibition.

The limit of detection (LOD) for sulcotrione, estimated as the concentration inducing 20% of inhibition, was calculated at $1.4.10^{-10}$ M. This LOD is significantly lower than the value obtained with the HPLC methods based on HGA determination ($0.15.10^{-6}$ M) [29] or based on direct β-triketone detection ($0.26.10^{-6}$ M) [60]. The described method is also much more sensitive than the previously reported whole-cell colorimetric assay (LOD = 38.10^{-9} M) [45]. The performance of the sensor is in good agreement with European Union legislation, which sets a maximum concentration of 0.1 μg L^{-1} for pesticides in drinking water, equivalent to 3.10^{-10} M for sulcotrione.

4. Conclusion

In this work, an amperometric HPPD-based biosensor was successfully applied for the monitoring of sulcotrione, a β-triketone herbicide, using a PEDOT-modified screen-printed graphite electrode that allowed obtaining higher current intensities and lower working potential. The decrease in the amperometric response after interaction of the HPPD with sulcotrione was used to determine sulcotrione concentration. Although numerous studies have been reported on this enzyme, it is the first time that optimal parameters of a purified HPPD were evaluated. HPPD parameters have been estimated using amperometry, results confirmed that HPPD has a good affinity for its substrate, and that sulcotrione is a reversible competitive inhibitor to this enzyme, showing a very high affinity for this enzyme. This biosensor showed a very high sensitivity for sulcotrione, with a LOD of $1.4.10^{-10}$ M, suggesting that this tool could be employed for the in situ monitoring. In future works, the biosensor performances will be enhanced by immobilization of the enzyme on the electrode surface.

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