Journal of Molecular Catalysis B: Enzymatic 72 (2011) 193–198

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Gallic acid interference on polyphenolic amperometric biosensing using *Trametes versicolor* laccase

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ARTICLE INFO

Article history:
Received 15 December 2010
Received in revised form 7 June 2011
Accepted 8 June 2011
Available online 15 June 2011

Keywords:
Gallic acid
Polyphenol
Trametes versicolor
Inhibitory activity
Amperometric biosensor

ABSTRACT

The present work reports the gallic acid (GA) interference on polyphenolic amperometric biosensing using *Trametes versicolor* laccase (TvLac). GA′ inhibitory effect on TvLac activity was investigated on the oxidation of caffeic acid (CA) by free TvLac and its immobilised form on modified polyethersulfone membrane (PES/TvLac), using spectrophotometric and amperometric biosensor detection methods. The results have indicated that GA presents inhibitory behaviour on TvLac activity in a concentration-dependent manner. The GA concentration leading to 50% activity lost, IC_{50}, was determined to be 19.15 ± 0.11 μM and 5.11 ± 0.19 μM for free and immobilised enzyme, respectively. The results have also shown that GA exhibited a competitive and a mixed inhibition types on the TvLac activity for spectrophotometric and amperometric biosensor methods, respectively. Further GA′ and CA′ cyclic voltammetry studies have demonstrated that GA′ oxidation products interfered with CA′ redox reaction products. In fact, a decrease of the reduction current was observed at cyclic voltammograms of CA, when mixed with GA. Therefore, the GA′ interference on polyphenolic amperometric biosensing is the result of the combination of two factors: on one hand, we have the inhibitory enzymatic effect, and on the other, the reaction of GA′ oxidation products with the o-quinones obtained by the enzymatic oxidation of CA. Both gave rise to the amperometric signal decreasing effect.

1. Introduction

Trametes versicolor belongs to the white-rot basidiomycetes fungi and produces an extracellular enzyme named laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) (TvLac) [1]. TvLac is a blue multicopper oxidase enzyme which uses molecular oxygen to oxidise phenolic and non-phenolic compounds [2,3]. In a typical laccase reaction (Scheme 1A) the phenolic substrate undergoes a one-electron oxidation generating a free radical (phenoxyradical). This active oxygen species, typically unstable, can be converted into a quinone in a second oxidation step [4].

Laccases are attractive for many biotechnological applications such as biosensors for analysis of phenolic compounds in blood, wine, tea, fruit juice, oil and medicinal herbs extracts [5]. In this context laccases from various sources have been successfully immobilised on various supports for use as biological recognition element of electrochemical biosensors ([1] and references therein). In the laccase-based amperometric biosensors, the quinones produced by the second stage of enzymatic oxidation can be electrochemically reduced to the initial phenolic compound, at electrode surfaces, at an appropriate potential (Scheme 1B). The measured current is proportional to the concentration of phenolic compounds.

Son et al. [6] have reported that certain phenolic acids inhibit tyrosinase (a polyphenol oxidase) activity by binding to the active site of the enzyme. These authors reported that gallic acid (GA; 3,4,5-trihydroxybenzoic acid) showed inhibitory activity on tyrosinase similar to ascorbic acid. Thus, GA has been described as an inhibitor of tyrosinase activity, and yet, GA has also been referred as a tyrosinase substrate, being oxidised by the enzyme [7,8]. It is known that TvLac belongs to the well studied laccases [9], however to our knowledge, there are no studies in the literature regarding TvLac inhibition by GA. Therefore, the purpose of the present work was to investigate the possibility of GA interference on polyphenolic amperometric biosensing using TvLac. In fact, GA is found in most foods and plants, being one of the most important simple polyphenolic units of tannins. Thus, GA′ inhibitory effect on TvLac activity was investigated on the oxidation of caffeic acid (CA) by free TvLac and its immobilised form on modified polyethersulfone membrane (PES/TvLac), using comparative spectrophotometric and amperometric biosensor detection methods.

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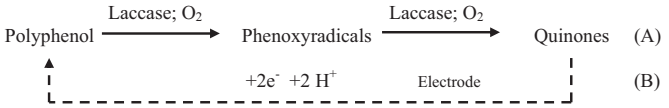
doi:10.1016/j.molcatb.2011.06.005



Scheme 1. Reaction of polyphenol substrate oxidation catalysed by laccase.

Subsequently, the electrochemical behaviour of CA and GA was also studied by cyclic voltammetry, in order to verify a possible interfering effect on the amperometric sensor system.

2. Materials and methods

2.1. Reagents and solutions

T. versicolor laccase (EC 1.10.3.2, 30.6 U mg^{-1}) was purchased from Fluka (Steinheim, Germany). Gallic acid, caffeic acid, catechin, chlorogenic acid, rutin, rosmarinic acid were obtained from Sigma–Aldrich (St. Louis, MO) (http://www.sigmaaldrich.com). L (+) tartaric acid was obtained from Riedel-deHaën (Seelze, Germany). Absolute ethanol, density 1.790 kg L^{-1} was obtained from Panreac Quimica AS (Barcelona, Spain) (http://www.panreac.es/). Polyethersulfone (PES) membranes (Ultrabind US450 0.45 μm) were from Gelman (http://www.pall.com). All other chemical reagents used were of analytical grade. All aqueous solutions were prepared using water purified with a Milli-Q A10 system (Millipore). Stock solutions of phenolic compounds (10 mg mL^{-1}) were prepared in ethanol. The assay solutions were prepared by diluting the stock solutions in 0.033 M tartrate buffer pH 3.5. This pH value was chosen because it is a value close to the average pH value of wines and the biosensor was also intended to be applied in wines. In fact it has been extensively studied and observed in our laboratory, that good results could be obtained with laccase immobilised at 4.5 and working in a pH 3.5 medium, as previously published [10].

2.2. TvLac immobilisation

The concentration of immobilised TvLac was fixed in all the experiments. The enzyme, laccase (TvLac) at 9.8 mg mL^{-1} solution, was prepared in acetate buffer of pH 4.5 and 10 μL (containing 0.1 mg of TvLac) was dropped on the polyethersulfone membrane disc of 18 mm diameter, with a micropipette. The enzyme was allowed to react with the membrane for 2 h at room temperature (ca. 22 °C). (In order to optimize the amount of laccase 1.10.3.2 which was retained on the membranes used, washings of membranes initially containing an excess of enzyme were done with acetate buffer. The presence of the enzyme which passed into the washing solutions was tested adding 12 μL of 50 mM ABTS solution to 2 mL of the washing solution and running a UV–vis spectrum. Washings were repeated until only one absorption peak, corresponding to ABTS (ca. 315 nm), was observed and the other peak corresponding to the ABTS oxidised species, the radical $ABTS^{\bullet -}$ in the region of 400 nm had disappeared. The latter mentioned observation meant that there was no more enzyme in excess in the washing solution. In this way, it was observed that 0.1 mg of laccase was the required amount of enzyme necessary to be immobilised on one membrane disc of 18 mm diameter) [10–12].

2.3. Amperometric detection of phenolic compounds

All the studies in this paper were carried out at least in triplicate and the operating conditions were set at 25 °C. In a previous paper [13], the development of a TvLac-based biosensor was presented. All measurements were performed using a 4208 electrode base sensor, Pt–Ag, AgCl from Universal Sensors (New Orleans, LA). The electrode base system consisted of platinum wires 0.3 mm diameter and silver, silver chloride helix which were contained in an electrode plastic cap closed on one end by an internal dialysis membrane. An amperometric biosensor detector (ABD) from Universal Sensors (New Orleans, USA) was used to apply the required potential and measure the consequent current obtained. The transients of current versus time were recorded on a Pharmacia Biotech recorder REC102. Amperometric measurements were done as follows: the bioelectrode was prepared by attaching the enzyme-modified membrane (PES/TvLac) to the electrode base system, with an o-ring. The bioelectrode was immersed in 5 mL of 0.033 M tartrate buffer solution pH 3.5, the potential was applied and, when the base current attained a stable value, 50 μL of substrate (phenolic) solution was directly added under continuous magnetic stirring at 300 rpm. The experiments were carried out at a constant potential (100 mV versus Ag/AgCl) and the current response corresponding to the reduction of enzymatically produced o-quinone was recorded as function of time.

2.4. Cyclic voltammetry

Cyclic voltammetry was performed using VoltaLab PGZ 100 from Radiometer Analytical connected to a PC software VoltaMaster 4 version 3.3. Experiments were carried out in a three electrode cell with 10 mL of tartrate buffer pH 3.5. The auxiliary electrode was a platinum wire, the reference was a saturated calomel electrode (SCE) from Schott Instruments and, the working electrode was a 3-mm glassy carbon disk (GCE) (BAS010420) (BAS Inc., Tokyo, Japan) which was cleaned by polishing in 0.05 μm alumina powder for 30 s and rinsed with deionised water in an ultrasonic bath for 3 min. In order to have electrochemical reproducible results the GCE was cleaned after each voltammogram experiment.

2.5. Spectroscopic and TvLac activity assays

All spectroscopic and spectrophotometric assays (free TvLac activity assays) were carried out on a UV-Vis double beam Shimadzu UV-1603 spectrometer (Shimadzu, Kyoto, Japan) connected to a PC (UVPC® Personal Spectroscopy Software). UV–visible absorption-spectroscopy studies were performed in 1 cm path cuvette and in 0.033 M tartrate buffer pH 3.5 containing 50 μM CA or 50 μM GA or mixture of both phenolics and TvLac solution (0.10 U).

All enzymatic reactions were performed in 0.033 M tartrate buffer pH 3.5. The final volume of all the reactions mixtures was 1.2 mL in a 3 mL, 1 cm width, UV–vis cuvette. The rate of the enzymatic oxidation reaction of CA and GA was determined spectrophotometrically by monitoring substrate consumption: CA (λ_{max} = 321 nm; $\varepsilon_{321\,nm}$ = 13,700 M^{-1} cm^{-1}, pH 3.5) and GA (λ_{max} = 270 nm; $\varepsilon_{270\,nm}$ = 9100 M^{-1} cm^{-1}, pH 3.5), respectively for 2 min. The activity assay mixture consisted of 100 μL enzyme solution (0.033 mg mL^{-1}), 100 μL of tartrate buffer pH 3.5 and 1000 μL of substrate solution. CA′ and GA′ concentrations ranged from 0.02 to 0.2 mM and 0.05 to 0.5 mM, respectively. The activity was obtained from the slope of the first 10 s of the graph and was calculated in international unit (U) which is the amount of TvLac that oxidises 1 μmol of substrate per min at 25 °C. The Michaelis–Menten constants for the substrates were obtained by regression of the measured values.

2.6. Inhibition studies

The inhibition kinetic assays were performed to determine the type of inhibition on free and immobilised TvLac (PES/TvLac) by GA. A typical enzyme inhibition assay was designed to determine the nature of the inhibition process. For this purpose, a differ-

Table 1
Amperometric detection of phenolic compounds.

Phenolic compounds[a]	Biosensor response, ΔI (nA)[b]
Individual	
1. Caffeic acid, 5 μM	−0.42 ± 0.02
2. Catechin, 5 μM	−0.28 ± 0.01
3. Chlorogenic acid, 5 μM	−0.29 ± 0.01
4. Rosmarinic acid, 5 μM	−0.21 ± 0.02
5. Rutin, 5 μM	−0.09 ± 0.01
6. Gallic acid, 50 μM	−0.04 ± 0.01
Mixture	
$M_1 = 1 + 2$	−0.71 ± 0.01
$M_2 = M_1 + 3$	−1.00 ± 0.03
$M_3 = M_2 + 4$	−1.20 ± 0.03
$M_4 = M_3 + 5$	−1.28 ± 0.02
$M_5 = M_4 + 6$	−0.05 ± 0.02
$M_0 = 1 + 6$	−0.02 ± 0.01

[a] Phenolic solutions in 0.033 M tartarate buffer, pH 3.5.
[b] Data are presented as means ± standard deviation of reduction current ($n = 3$).

ent set of assays was carried out at constant concentration of GA (5–10 μM) and varying the concentration of CA (0.02–0.2 mM). The activity assay was performed as described above, replacing 100 μL of tartrate buffer pH 3.5 by 100 μL of inhibitor solution and, monitoring the associate decrease in absorbance at 321 nm. This decrease is proportional to the rate of CA consumption. The assays for immobilised TvLac (PES/TvLac) were performed amperometrically, as described above, and initial velocities (nA min^{-1}) were determined at CA concentration in the absence of gallic, as well as with added GA at concentrations ranging from 5 to 10 μM. To assess the GA concentration needed to reduce TvLac activity by 50% (IC_{50}), the experiments were carried out at constant concentration of CA (0.015 mM) and varying the concentration of GA (1–150 μM) and (1–30 μM) for free and immobilised TvLac, respectively. A control reaction was carried out using 0.015 mM CA without inhibitor. The inhibition percentage was calculated by means of the formula:

$$I = 100 - \left(\frac{V_{i,\text{sample}}}{V_{i,\text{control}}}\right) \times 100$$

where I is the inhibition (%), $V_{i,\text{sample}}$ is the initial oxidation rate of the sample and V_{control} is the initial oxidation rate in control test. The IC_{50} values were obtained from the plot of the logarithm of GA concentrations against the percent inhibition of the enzyme activities.

3. Results and discussion

3.1. GA′ negative synergetic effect on the biosensing reduction current

The influence of phenolics interactions on the biosensing reduction current was studied for six compounds: caffeic acid, catechin, chlorogenic acid, rosmarinic acid, rutin and gallic acid. Each phenolic compound was at a concentration of 5 μM, except GA that was at 50 μM.

Firstly, the possibility of phenolic compounds redox interactions at the electrode surface was investigated. It was observed that the electrode base system did not respond to those compounds at the applied potential studied. Afterwards, each individual compound and successive combined mixed solution was analyzed using the amperometric biosensing system for phenolic detection. That assay consisted of measuring the current signal variation (ΔI (nA)) corresponding to the reduction of enzymatically produced o-quinones at the electrode, as shown in Scheme 1. Table 1 shows the effect of phenolic compounds on the biosensor response in terms of reduction current (nA). The above results have indicated that phenolic mixtures presented two different effects on the biosensing response. Four mixtures (M_1, M_2, M_3 and M_4) showed an additive reduction current behaviour resulting on the summation of individual reduction currents.

However, the addition of mixture M_5, containing GA, led to a decrease on biosensing detection. Thus, GA has demonstrated a negative synergetic effect on the biosensing reduction current. It must be stressed that the biosensor did not respond to a 5 μM GA solution on itself. Possibly, GA′ negative synergetic behaviour may be attributed to chemical interactions among phenolic molecules, a lower TvLac specificity towards GA and/or an inhibitory effect on TvLac activity.

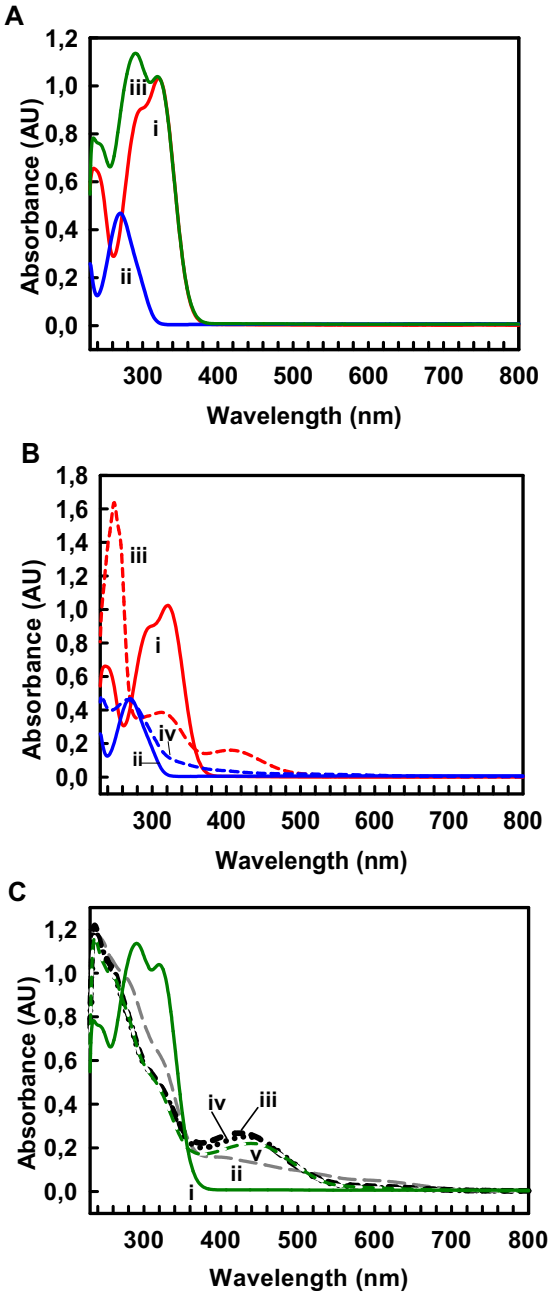


Fig. 1. UV–vis absorbance spectra at pH 3.5. Initial conditions: caffeic acid (50 μM); gallic acid (50 μM) and TvLac (0.10 U). (A) Caffeic acid (i), gallic acid (ii), and a mixture of both phenolic compounds (iii); (B) caffeic acid (i) and gallic acid (ii) oxidation by TvLac and respective enzymatically oxidised end products: (iii) from (i); and (iv) from (ii), at 2 min of reaction; (c) mixture of caffeic acid and gallic acid oxidation by TvLac at five instants: (i) 0 min, (ii) 2 min, (iii) 4 min, (iv) 6 min, and (v) 8 min of enzymatic reaction.

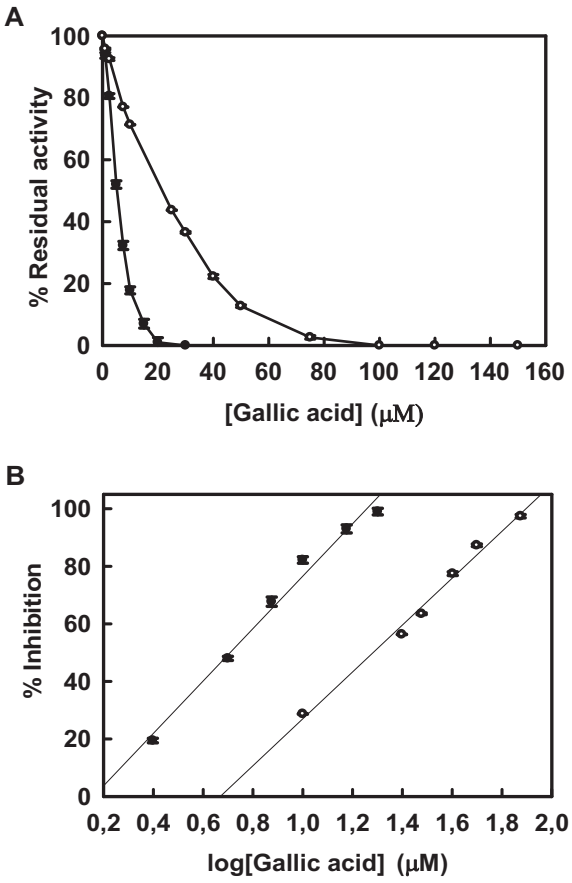


Fig. 2. (A) Effect of gallic acid on the activity of TvLac. (B) Inhibition curves for gallic acid. Caffeic acid (0.015 mM) was used as substrate; free TvLac (spectrophotometric method) (○) and immobilised (PES/TvLac) (amperometric biosensor method) (●).

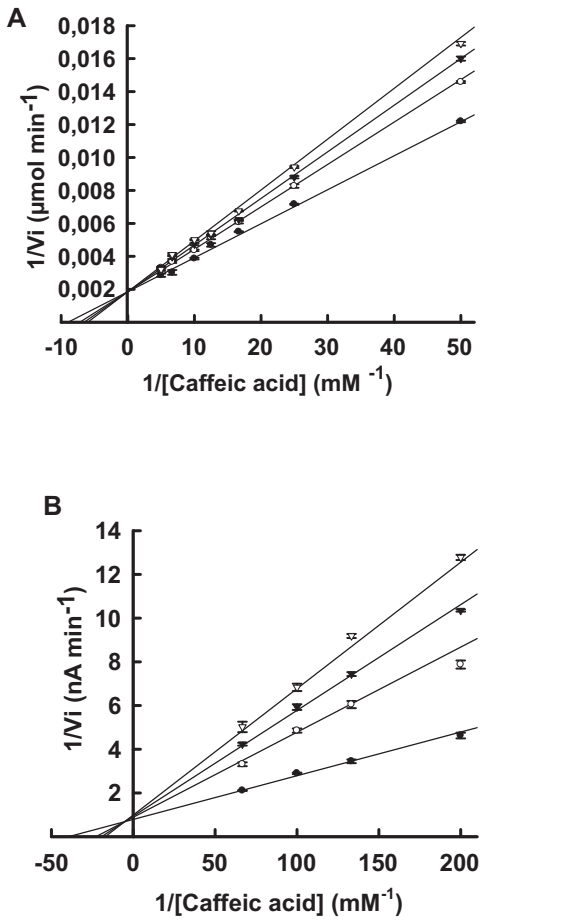


Fig. 3. Lineweaver–Burk double reciprocal plots showing inhibition of TvLac by gallic acid: (●) control, (○) 5 μM, (▼) 7.5 μM, and (▽) 10 μM. Caffeic acid was used as substrate. (A) Free TvLac (spectrophotometric method) and (B) immobilised (PES/TvLac) (amperometric biosensor method).

In order to investigate these possibilities, GA inhibitory enzymatic activity studies were performed with spectrophotometric UV–vis detection for free TvLac. Parallel studies were done for immobilised TvLac on modified PES membranes (PES/TvLac) with amperometric detection—that was the biosensor case, and following the redox response by cyclic voltammetry.

CA and GA were used in the subsequent experiments. First of all, the reason of this choice was due to the fact that CA (3,4-dihydroxycinnamic acid) and GA (3,4,5-trihydroxybenzoic acid) are structurally similar, both have a catechol skeleton, though GA has an additional hydroxyl group as shown in Table 2. Additionally, CA was the phenolic compound studied in this work that presented the highest biosensing reduction current as shown in Table 1. Moreover, the mixture of CA (5 μM) and GA (50 μM) (M_0) exhibited a strong negative synergism effect as shown in Table 1.

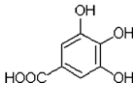
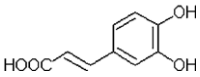
3.2. UV–visible absorption-spectroscopy studies

UV–visible absorption-spectroscopy studies were carried out in order to observe the effects of the chemical interaction among phenolic molecules and its corresponding enzymatically oxidised products.

Preliminary studies were done following the absorption spectra from control solutions containing only CA, GA and a mixture of both phenolic compounds, for 30 min. Fig. 1A shows the corresponding overlayed UV–vis spectra at pH 3.5. The absorption spectra of the mixed solution showed no variations with respect to the summation of the spectra of the separate compounds, thus suggesting that no interactions and/or autoxidation occurred in a 30-min period for individual and combined phenolics. The results showed that the absorption spectra of CA and GA solutions had an absorbance maximum at 321 nm and 270 nm, respectively. These data are in accordance with those reported in the literature [14,15].

Enzymatic oxidation of CA, GA and a mixture of both phenolic acids, by TvLac, was followed by UV–vis absorption at pH 3.5, and the respective spectra are displayed in Fig. 1B and C. Fig. 1B shows that enzymatic oxidation has been characterised by the appearance of a new peak with the maximum at 405 nm and 385 nm for CA and GA respectively. Those news peaks presumably corresponded to the formed o-quinone absorption [7,16]. Fig. 1B shows that TvLac oxidised both compounds and also, apparently, seemed to have a higher action on CA, giving rise to the corresponding o-quinone, which exhibited a maximum absorbance at 405 nm. However, when CA was oxidised in the presence of GA, the maximum absorbance was shifted up to 450 nm, resulting in a large band with maximum absorbance around 430–450 nm as shown in Fig. 1C. The enzymatic oxidation of the phenolic acids results in their corresponding o-quinones, which then undergo non-enzymatic reactions, generating polymeric phenolic compounds, since they are reactive oxidizing agents and electrophiles. It is often suggested that brown pigments may have been due to the oxidative polymerisation reactions induced by the o-quinone oxidation product [7,17]. The spectroscopic studies evidenced that o-quinones resulting from phenolic acids, were rather unstable, which is a characteristic behaviour of many o-quinones. In fact, a decrease of the maximum of absorbance band around 430–450 nm, as function of time, was observed confirming the enzymatically oxidised end products disappearance, as shown in Fig. 1C (iii–v).

Table 2
Substrate specificity of TvLac.

Substrate	K_m [a] (mM)	V_{max} [a] (μmol mg^{-1} s^{-1})	V_{max}/K_m (mg^{-1} L^{-1} s^{-1})	Specificity[b] (%)
Gallic acid 	0.53 ± 0.05	8 ± 0.4	0.015 ± 0.006	2.12
Caffeic acid 	0.11 ± 0.02	78 ± 5	0.709 ± 0.016	100

[a] K_m, Michaelis–Menten saturation constant; V_{max}, maximum reaction rate of oxidation. Data was obtained from Lineweaver–Burk plots where x intercept = $-1/K_m$, and y intercept = $1/V_{max}$. The data are presented as means ± standard deviation (n = 3).
[b] The catalysis efficiency was defined as ratio of V_{max} to K_m, the percentage of specificity was calculated using caffeic acid as reference.

3.3. TvLac substrate specificity

Our spectroscopic studies showed, as expected, that GA and CA were oxidised by TvLac. The kinetic parameters of enzyme biocatalysis using GA and CA as substrates were studied to elucidate about TvLac substrate specificity.

Michaelis–Menten kinetics between the substrate concentration and the initial rate of the TvLac oxidation reaction has been found [18]. Table 2 shows the kinetic parameters obtained from a Lineweaver–Burk plot: the Michaelis–Menten constant (K_m) and the maximum reaction rate (V_{max}). K_m is the parameter that shows the affinity of enzyme towards substrate. Lower K_m value means higher affinity of the enzyme to the substrate. We found that TvLac had an almost 5-fold higher affinity to CA (K_m = 0.11 ± 0.02 mM) than to GA (K_m = 0.53 ± 0.05 mM).

The catalytic efficiency expressed as the V_{max}/K_m ratio, was also used as an indication of the ability of TvLac to convert substrates into corresponding end products. TvLac catalytic efficiency for CA was 47 times greater than for GA as shown in Table 2. Some authors [6,19,20] have also reported that CA was more efficiently oxidised than GA by enzymes such as tyrosinase and laccase.

3.4. TvLac activity inhibition by GA

The effect of GA on oxidative reaction of CA was studied on free and immobilised TvLac (PES/TvLac) using spectrophotometric and amperometric biosensor methods, respectively. The relationship between residual enzyme activity and the concentration of GA is shown in Fig. 2A. The result shows that GA inhibited TvLac activity and that inhibition was concentration dependent. In fact, the residual TvLac activity decreased as the concentration of GA increased.

In this study, the concentration of GA that decreased the rate of the enzymatic oxidation reaction of CA by 50% (IC_{50}) was obtained plotting the inhibition percentage against the logarithm of the concentration of GA solution as shown in Fig. 2B. The free TvLac assay was less sensitive towards GA inhibition (IC_{50} = 19.15 ± 0.11 μM) than the TvLac immobilised on modified PES membranes (IC_{50} = 5.11 ± 0.19 μM). These results indicated that GA inhibition was approximately four times more effective on immobilised enzyme.

Furthermore, the inhibition kinetics of GA on free and immobilised TvLac (PES/TvLac) were analyzed by Lineweaver–Burk plots as shown in Fig. 3. The four lines were obtained from the uninhibited enzyme activity on CA and from three different concentrations of GA (5, 7.5 and 10 μM). For free TvLac the lines intersect on the vertical axis as shown in Fig. 3A. This result indicated that GA exhibited a competitive inhibition for CA oxidation by TvLac. However, immobilised PES/TvLac exhibited a mixed inhibition, as revealed in Fig. 3B. These findings indicated that PES/TvLac associated with amperometric biosensor method provided a more favourable microenvironment to GA inhibitory activity than free enzyme.

3.5. Cyclic voltammetry studies

It is important to notice that polyphenols are redox cycled between enzymatic oxidation and electrochemical reduction giving a response at TvLac modified electrode. The responses should be proportional to the concentration of the phenolic compounds in the solution, if they are meant to have analytical application. In order to better understand the electrochemical behaviour of GA and CA, some experiments were performed using cycling voltammetry at GCE, in the range of 200–600 mV (versus SCE), at a scan rate of 100 mV s^{-1}. The pH value of the solutions was 3.5.

The cyclic voltammogram (CV) of 50 μM CA is shown in Fig. 4A. The anodic peak potential (E_{pa}) was at 448 mV, which corresponds to the oxidation of the phenolic hydroxyl groups of CA, and the cathodic peak potential (E_{pc}) at 416 mV, which corresponds to the reduction of the formed o-quinone. The separation between the anodic and cathodic peak potential, $\Delta E_p = |E_{pc} - E_{pa}|$ = 32 mV, and the ratio of the anodic peak current to the cathodic one, I_{pc}/I_{pa} = 0.93, confirmed that the electrochemical process of CA at a GCE was reversible under these conditions.

Curves a–f in Fig. 4B show the cyclic voltammograms of 12.5; 25; 50; 125; 250 and 500 μM GA solutions, respectively. Cyclic voltammetric experiments showed a single oxidation peak at 470 mV, very similar in size and shape to that produced by CA [16,21], and on the reverse scan, no reduction peak was observed, showing that GA was irreversibly oxidised at the GCE, suggesting an oxidation process coupled with a fast subsequent chemical reaction [21].

The CV's showed successive enhancement of peak current on increasing GA concentration. The plot of peak current versus the respective concentration of GA was found to be linear in the range 12.5–500 μM, I_{pa} $(GA)_{experimental}$ = 0.0037 [GA] + 0.1383; R^2 = 0.9934 as shown in Fig. 4D.

The oxidation of 50 μM CA to the corresponding o-quinone, in the presence of GA (0–500 μM) was also studied in order to evaluate the effect of GA on electrochemical behaviour of CA. Curves a–g in Fig. 4C showed the correspondent CV's. The experimental anodic peak current of the mixed solutions varied with GA concentration, but the corresponding slope was smaller than that expected for the theoretical anodic peak current variation (the sum of the peak currents of the two separate voltammograms), that is:

$$I_{pa}\,(\mathrm{CA{+}GA})_{experimental} = 0.0029\,[\mathrm{GA}] + 0.5024; \quad R^2 = 0.998$$

and

$$I_{pa}\,(\mathrm{CA} + \mathrm{GA})_{theoretical} = 0.0038\,[\mathrm{GA}] + 0.5794;\ R^2 = 0.9895.$$

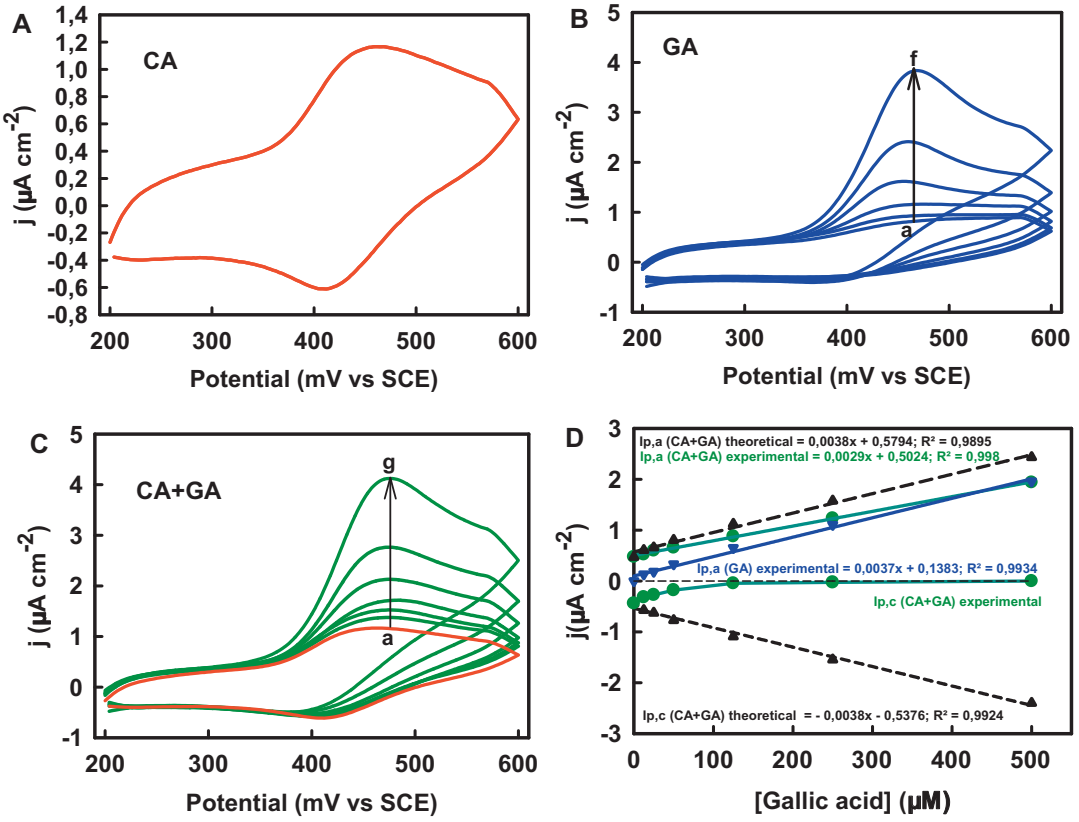


Fig. 4. Cyclic voltammograms at pH 3.5 were obtained in the range of 200–600 mV at scan rate of 100 mV s^{-1}. WE, 3 mm GCE. (A) Caffeic acid (50 μM); (B) gallic acid (12.5–500 μM) corresponding to a–f, respectively; (C) mixture of both phenolic compounds at fixed concentration of caffeic acid (50 μM) and varying the gallic acid concentration from 0 to 500 μM, corresponding to a–g, respectively; (D) current peak versus gallic acid concentration from 12.5 to 500 μM, and at fixed concentration of caffeic acid (50 μM) with respective gallic acid concentration from 0 to 500 μM.

The experimental findings showed that, when the GA concentration increased, I_{pa} (CA + GA) approached I_{pa} (GA) indicating the total CA disappearance, as shown in Fig. 4D. On the reverse scan, the I_{pc} $(CA + GA)_{experimental}$ results proved that the irreversibility of redox system process for the mixture CA + GA increased with GA concentration, that is, the cathodic current values rapidly decreased with GA concentration and approached zero for 125 μM GA.

The present results have demonstrated that the products of the oxidation and subsequent chemical reactions of GA in the mixture CA + GA, rapidly removed the generated products of the oxidation of CA and, moreover reduced the CA concentration at the electrode surface as above mentioned.

4. Conclusions

The initially observed GA′ negative synergetic effect on biosensing amperometric detection may be attributed to GA′ inhibitory effect on the TvLac activity. In fact, the results presented in this work have demonstrated that GA reduces the TvLac-activity in free and immobilised PES/TvLac. Additionally, it has been demonstrated that GA′ oxidation and subsequent chemical reaction products, interfered with CA oxidation products, contributing to the effective biosensor signal reduction due to the disappearance of enzymatically oxidised end products.

Acknowledgements

D.M.A. Gil acknowledges FCT (Fundação para a Ciência e a Tecnologia) for a Ph.D. fellowship (SFRH/BD/34511/2006). The authors acknowledge Financial support from Faculdade de Ciências da Universidade de Lisboa.

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