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Electrocatalytic oxidation of ascorbate by heme-Fe^{III}/heme-Fe^{II} redox couple of the HRP and its effect on the electrochemical behaviour of an L-lactate biosensor

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Abstract

The measurements of L-lactate using the carbon paste electrode modified with lactate oxidase (LOD), horseradish peroxidase (HRP) and ferrocene (FcH) operating at low working potential in flow injection mode showed that the intensity as well as the shape of peaks were dependent on the concentration of the reducing species present in samples (e.g. ascorbate) even at low operating potentials (− 200 to 0 mV vs. Ag/AgCl). The mechanism of the electrochemical contribution of ascorbate to the L-lactate response was examined by using cyclic voltammetry, hydrodynamic voltammetry and FIA results. Comparative studies showed that HRP was catalytically active for the oxidation of ascorbate leading to a decrease in the cathodic electrochemical signal of L-lactate. The results of our investigation postulated that the direct electron transfer from the HRP-Fe(III)/HRP-Fe(II) redox couple to the electrode surface was involved in the electrocatalytic oxidation of ascorbate at the electrode surface.

Keywords: Biosensor; Carbon paste electrode; Horseradish peroxidase; Direct electron transfer; Ascorbate

1. Introduction

The electrochemical determination of L-lactic acid using electrodes modified with enzyme has received much attention due to its importance in food fields [1,2]. In several cases, an accurate measurement of the concentration of L-lactic acid needs a sensitive and selective enzyme electrode, mainly in a complex matrix with low level of lactic acid. Indeed, the determination of low concentrations in complex matrix can be affected by various interfering species that could contribute directly or indirectly to the amperometric response [3]. For example, food products based on fruits or vegetables can be typical complex matrix that contains a low amount of L-lactic acid compared to one of the interfering species such as ascorbic acid (AA). In such media, ascorbic acid can be present naturally or added as antioxidant agent [4].

Among the ways described to circumvent the problem of electrochemical interferences, one consisted in removing ascorbic acid [5] by using enzymes such as ascorbate oxidase (AsOD) directly added into the sample or immobilised in a pre-reactor [6,7] or at the electrode surface. The use of a selective membrane to reduce the diffusion of ascorbic acid to the electrode surface has also been suggested [8,9]. However, this latter procedure has been found not effective when the ascorbic acid and L-lactic acid are present in comparable concentration in the same sample.

Another way reported in the literature to minimise electrochemical interferences consisted in applying low value of working potential. It was found that a potential range between − 200 and 0 mV vs. Ag/AgCl seems more suitable for biosensors [2,10] based on the measurement of hydrogen peroxide. In these biosensors, horseradish peroxidase (HRP) was employed to detect H_2O_2 produced by oxidase reactions [11–13]. However, when dealing with measurements in real samples, the presence of various electroactive species could influence the biosensor performances even at low operating potentials. Indeed, studies

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concerning the selectivity of the L-lactate biosensor based on LOD and HRP [11] showed that the measurements of L-lactate concentrations using FIA mode were found significantly affected by the presence of ascorbate even at a potential of − 50 mV vs. Ag/AgCl.

On the other hand, it is well known that the unmediated electron transfer of HRP is assumed to be kinetically slow compared with the mediated one. It is obvious that the critical step in improving both sensitivity and selectivity of LOD/HRP-modified carbon paste electrode is the effective regeneration of the active form of HRP. The effect of a mediator on the analytical performances of the bienzymatic GOD/HRP electrode has already been studied, and it has been demonstrated that the introduction of ferrocene significantly increased the amperometric response [14]. In this study, the use of ferrocene in the LOD/HRP-modified CPE was also found beneficial in terms of sensitivity because the highest response with mediated electron transfer of HRP was obtained. The ratio of mediated to unmediated electrooxidation of L-lactate was found around 3–4 when L-lactate solution of 100 μM/l was injected.

In the present work, we studied and examined the effect of ascorbate on L-lactate determination. We simulated samples in which L-lactate and ascorbate are present in similar concentrations. Thus, the use of ferrocene in the preparation of HRP/LOD-modified CPE would increase the sensitivity and improve the limit of detection as well. Even if the biosensor used here did not present significant aspects of novelty since it is based on existing technology [11,15], the aim of this study was to examine how ascorbate could influence the electrochemical response of the LOD/HRP/FcH-modified CPE at low operating potentials. The results of our investigation provided a new and complementary information relevant to the electrochemistry of the Fe^{III}/Fe^{II} redox couple of HRP towards the catalytic oxidation of ascorbate.

2. Experimental

2.1. Reagents

Lactate oxidase (LOD) (EC. 1.1.3.4) was a Boehringer-Mannheim product. Horseradish peroxidase (HRP) (EC. 1.11.1.7) was from Roche. Ferrocene (FcH) (No. 46260), ascorbic acid (AA) (No. A-7506) and hydrogen peroxide (30% (w/w)) (No. H-1009) were obtained from Sigma. Paraffin oil (No. 7161) was purchased from Merck and graphite powder (No. 50870) from Fluka. L-Lactate and ascorbate aqueous solutions were prepared using 0.1 M phosphate buffer (pH 7.2) containing 0.1 M KCl as support electrolyte. Diluted samples were prepared by suitable dilution with the phosphate buffer solution.

2.2. L-Lactate biosensor: preparation and analytical performances

Carbon paste electrodes (CPEs) modified with LOD, HRP and FcH as redox mediator were prepared by a procedure similar to the one already used for GOD/HRP/FcH [14]. Briefly, HRP was oxidised by an aqueous solution of sodium periodate ($NaIO_4$). The oxidised enzyme was dissolved in 0.05 M phosphate buffer (pH 8). The graphite powder was then added. After 1 h mixing, the LOD suspension in water was added. The final mixture was freeze-dried. The enzymatic carbon paste was obtained by mixing in a mortar the dry enzymatic powder with paraffin oil in which ferrocene had already been dissolved in amount of 1.32% (w/w). LOD and HRP loadings in carbon paste were 2.0 and 13.6 U mg^{-1}, respectively.

The main reactions involved in the L-lactate detection are summarised in Fig. 1. Hydrogen peroxide produced enzymatically by LOD in the presence of L-lactate and molecular oxygen is oxidised at the electrode surface via HRP/FcH at low operating potential [13].

2.3. Apparatus and procedures

FIA measurements were performed using a three-electrode flow-through amperometric homemade cell of the wall-jet type containing a stainless steel wire as auxiliary and a solid Ag/AgCl as reference electrode which potential was taken as 217 mV vs. NHE. The cell was connected to a potentiostat (BAS model Petit Ampère CV-1B). A strip chart recorder (Linseis model L200E) was used to follow the electrode response. The flow-injection system consisted in a flow carrier and sample pump (Ismatec) and an electrical 6-way valve (Rheodyne) for the injections of the samples by means of a 50-μl injection loop. The flow

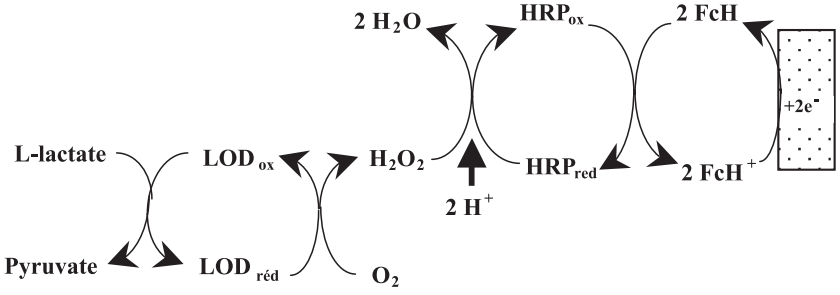

Fig. 1. Principle of electroenzymatic detection of L-lactate.

carrier, 0.1 M phosphate buffer (pH 7.2) added with 0.1 M KCl, was pumped at a flow rate of 0.7 ml min^{-1}. All presented results were the mean of at least three injections.

Cyclic voltammetry (CV) was carried out with the Powerlab electrochemistry workstation (ADInstruments) connected with a PC. A three-electrode cell including CPE as working electrode and solid Ag/AgCl as reference was also used. Phosphate buffers were purged with ARGON for about 15 min prior to a series of experiments. All measurements were performed at room temperature.

2.4. Dehydro-L-ascorbic acid/L-ascorbic acid formal redox potential: potentiometric determination

The formal redox potential of dehydro-L-ascorbic acid/L-ascorbic acid system ($E^{\circ\prime}_{DAA/AA}$) in our experimental conditions (i.e. 0.1 M phosphate buffer, pH = 7.2, containing 0.1 M KCl) was determined by potentiometric titration of 50 ml of 0.01 M solution of ascorbate by 0.05 M iodine solution. The experiment was performed using a bare CPE and a saturated calomel electrode (SCE) coupled with Methrom potentiometer E632. Results are shown in Fig. 2. By modelling experimental points with Nernst law, $E^{\circ\prime}_{DAA/AA}$ was found to be equal to -160 ± 10 mV vs. SCE (i.e. -136 ± 10 mV vs. Ag/AgCl). This result is coherent with data found in the literature for physiological pH [16].

3. Results and discussion

3.1. Ascorbate interferences evidence

The electrochemical response of LOD/HRP/FcH-modified CPE was examined in FIA mode by injecting an L-lactate solution in the presence and absence of ascorbate. In this study, ascorbate was used as an example of interfering species. A sharp and well-defined cathodic peak was obtained when the L-lactate solution prepared without ascorbate was injected (Fig. 3A). In contrast, the injection of the L-lactate solution containing ascorbate led to a significant change in the current–time profiles (Fig. 3B–D). The comparison between the peak intensities obtained in Fig. 3A and B showed that the presence of 80 μM of ascorbate in the L-lactate solution induced a decrease of about 30% in the peak intensity.

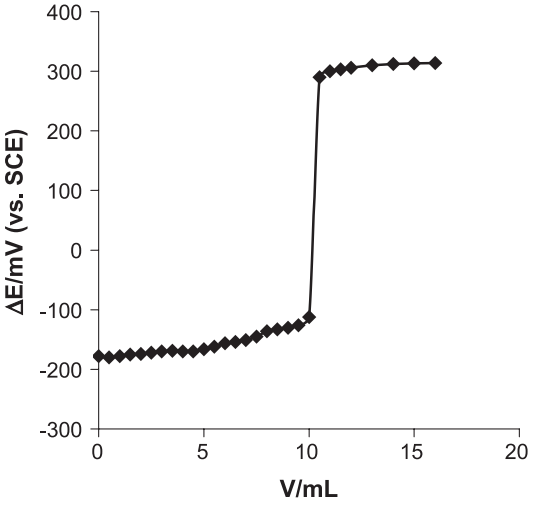

Fig. 2. Potentiometric titration of 50 ml of 0.01 M ascorbate solution in 0.1 M phosphate buffer, pH = 7.2, containing 0.1 M KCl.

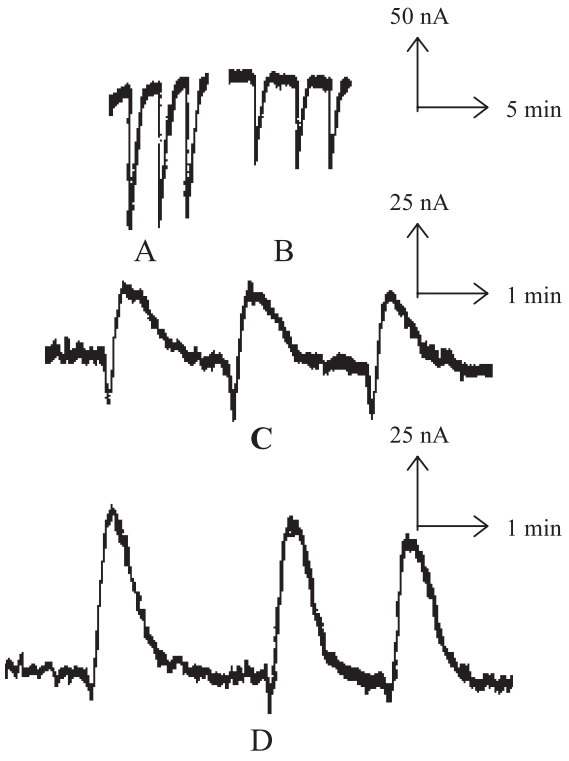

Fig. 3. Current–time profiles. LOD/HRP/FcH-modified carbon paste electrode. Working potential: −50 mV vs. Ag/AgCl. Flow rate: 0.7 ml min^{-1}. Phosphate buffer 0.1 M, pH 7.2. A: 40 μM L-lactate; B: A + 80 μM ascorbate; C: A + 240 μM ascorbate; D: A + 320 μM ascorbate.

The additional experiments showed that higher amounts of ascorbate in the L-lactate solution led not only to a decrease in peak intensities, but induced also a change in the shape of the current–time curves with the appearance of a broad anodic peak (Fig. 3C). When the amount of ascorbate was increased up to 320 μM in the L-lactate solution, the electrode response showed an almost disappearance of the cathodic peak to the advantage of the anodic one (Fig. 3D). However, no electrochemical response was obtained with a bare CPE when the same solutions were injected under the same experimental conditions. At this point, one can conclude that one or various components (HRP, LOD and FcH) used in the preparation of the L-lactate electrode could be involved in the electrochemical behaviour observed in the presence of ascorbate.

Therefore, hydrodynamic voltammograms (Fig. 4) were used to examine the amperometric response towards ascorbate solution successively at a bare CPE (a) and electrodes

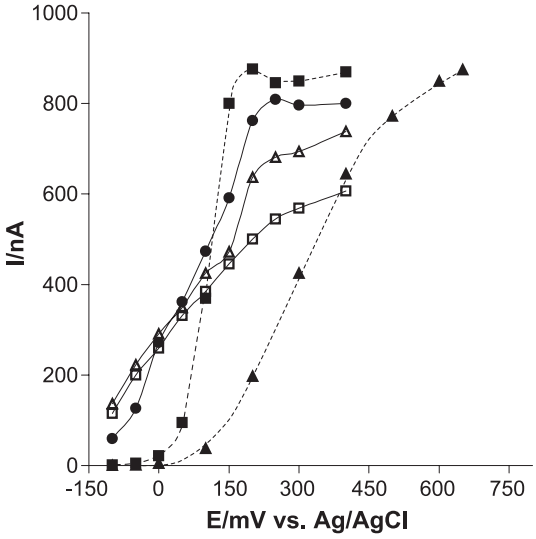

Fig. 4. Hydrodynamic voltammograms of ascorbate (200 μM) on (a: ▲) bare, (b: ■) FcH, (c: □) HRP, (d: △) LOD/HRP and (e: ●) LOD/HRP/FcH-modified carbon paste electrodes. Flow rate: 0.7 ml min^{-1}. Phosphate buffer 0.1 M (0.1 M KCl), pH 7.2.

modified with (b) FcH, (c) HRP, (d) LOD/HRP and (e) LOD/HRP/FcH. Fig. 4a shows that, at the bare CPE, the oxidation of ascorbate became significant only for working potential values higher than +150 mV vs. Ag/AgCl. The addition of ferrocene into the carbon paste led, as it was expected, to an electrocatalytic oxidation behaviour of ascorbate by ferricinium cation produced electrochemically at the electrode surface [17,18], but no significant anodic current was generated in the potential range of −150 to 0 mV vs. Ag/AgCl (Fig. 4b). By comparing the hydrodynamic voltammograms obtained in the presence (Fig. 4c–e) and the absence (Fig. 4a,b) of HRP into CPEs, it was found that all the HRP-containing electrodes exhibited a significant anodic current in a range of potential between −150 and 0 mV vs. Ag/AgCl when ascorbate solution was injected. It is therefore obvious that HRP is involved in the resulting electrochemical behaviour of ascorbate, and this was found independent on the presence or absence of FcH. On the basis of this observation, the unmediated electron transfer of HRP should be involved and make possible the electro-oxidation of ascorbate. Then, more experiments were achieved in order to examine the electrochemical behaviour of the HRP-modified CPE in the presence and absence of ascorbate.

3.2. Electrochemical behaviour of HRP-modified CPE in the presence and the absence of ascorbate

To attempt to explain the involvement of unmediated electron transfer of HRP in the electrochemical behaviour of ascorbate, a CPE modified with HRP only was examined by cyclic voltammetry in deoxygenated phosphate buffer solution. In the absence of ascorbate, the voltammogram of HRP-modified CPE exhibited a quasi-reversible electrochemical process (Fig. 5) centred at $E^{\circ\prime} = -170 \pm 40$ mV vs. Ag/AgCl with anodic and cathodic peaks around −40 and −300 mV vs. Ag/AgCl, respectively. This pair of peaks could be attributed to the direct electron transfer between HRP, the heme group of immobilised HRP and the graphite of modified CPE. No voltammetric response was observed at a bare CPE under the same experimental conditions. The ratio of cathodic to anodic peak currents was nearly unity. Peak currents varied linearly with the scan rate, indicating electrode process is not diffusion-controlled, as expected for an immobilized system. The peak-to-peak separation was more than 200 mV and the anodic and cathodic peak potentials shifted slightly in opposite directions with a change in scan rate which is consistent with a quasi-reversible electrochemical behaviour [19].

The direct electron transfer between HRP and carbon electrode has already been observed and the authors themselves noted an electrocatalytic process in the presence of H_2O_2 [20–22]. It was also demonstrated that HRP adsorbed on graphite [23,24], carbon nanotube [25], gold [26] or platinum [27] electrodes could exhibit catalytic response towards hydrogen peroxide. On the whole, the direct electron transfer was found enhanced when HRP was entrapped in a membrane [28–30], or confined in stable polymer films [31–33], in silica gel [34] or in DNA film [35]. The authors pointed out that, in the absence of hydrogen peroxide, the quasi-reversible electrochemical process of HRP was characteristic of the Fe^{III}/Fe^{II} redox couple contained in the HRP-heme.

In the present study, the cyclic voltammetry showed that HRP exhibited direct electron transfer without using any specific "promotor" or electrochemical treatment. Also, in order to examine that the resulting voltammogram did not result from a heme-Fe^{III}/heme-Fe^{II} redox system not linked to the protein, HRP was previously ultra-filtrated (cutoff 30 kDa) and its electrochemical behaviour was then examined in CPE. No significant difference was obtained between the

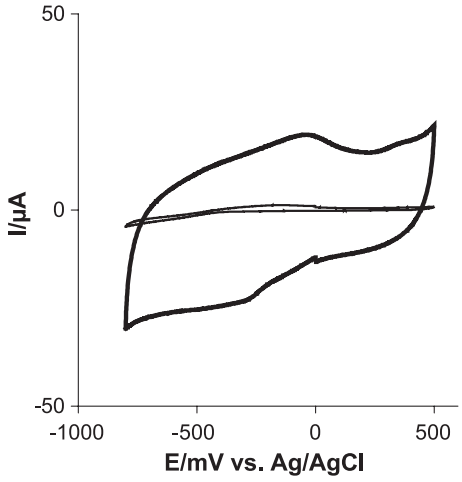

Fig. 5. Cyclic voltammogram of HRP-modified CPE (broad line) and bare CPE (slight line) in pH 7.2 deoxygenated phosphate buffer (0.1 M containing 0.1 M KCl) at room temperature. Scan rate 50 mV s^{-1}.

voltammograms (not shown) carried out on modified CPEs prepared with filtrated and unfiltrated HRP. The details of the direct electron transfer between enzyme (prosthetic group) and the electrode surface remained not completely understood so far. However, our result could plausibly be explained by the nature of the graphite used in this study [36]. It was noticed several times that the presence of functional groups (e.g. C=O, phenolic, quinoidal, etc.) on graphite surface could mediate electron transfer between enzyme and the electrode [20,37].

In additional experiments, the HRP-modified CPE showed that a catalytic behaviour towards a deoxygenated H_2O_2 solution was obtained (Fig. 6), and no electrochemical signal was obtained when the voltammogram was performed on bare CPE under the same experimental conditions. It is well known that the mechanism of direct electrocatalytic reduction of hydrogen peroxide at HRP-modified electrode involves two compounds noted communally compound-I and compound-II. This mechanism could schematically be described by the following reactions [38]:

$$\text{HRP-Fe(III)} + H_2O_2 \rightarrow \text{Compound-I} + H_2O \quad (1)$$

$$\text{Compound-I} + 1e^- \rightarrow \text{Compound-II (at electrode)} \quad (2)$$

$$\text{Compound-II} + 2H^+ + 1e^- \rightarrow \text{HRP-Fe(III)} + H_2O \text{ (at electrode)} \quad (3)$$

where compound-I has a +V oxidation state (Fe^{IV}=O,P^+), P^+ corresponding to a cation radical localised on porphyrin,

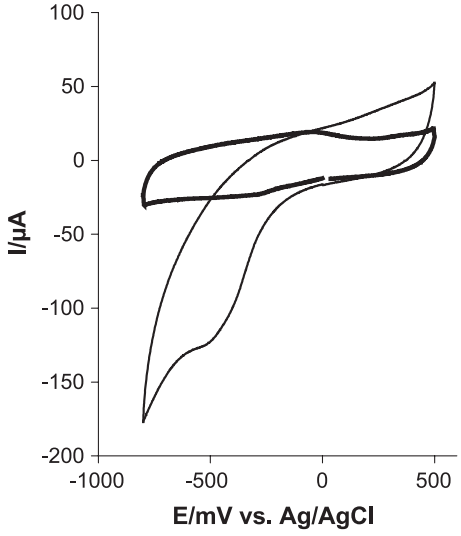

Fig. 6. Cyclic voltammogram of HRP-modified CPE in pH 7.2 deoxygenated phosphate buffer (0.1 M containing 0.1 M KCl) at room temperature in the absence (broad line) and presence (slight line) of 30 mM H_2O_2. Scan rate 50 mV s^{-1}.

and compound-II has +IV oxidation state (Fe^{IV}=O). It was reported that the electron transfer from compound-I to compound-II is considered as rapid, while the second transfer from compound-II to HRP is supposed to be the limiting step in the direct electrochemical reduction process of HRP [21]. The direct electron transfer from HRP to the electrode surface had been already observed using amperometric technique when the potential is set up between 200 and −200 mV vs. Ag/AgCl. Thus, the catalytic behaviour observed in Fig. 6 could result from the electrochemical regeneration of HRP-Fe(III) via compound-I and compound-II with a catalytic reduction of hydrogen peroxide. In the more negative direction of potential, HRP-Fe(III)-could directly be reduced at the electrode surface to HRP-Fe(II).

By taking into account the catalytic mechanism described above, the ascorbate should act as reducing agent [21] and could compete significantly with a direct or mediated electrochemical regeneration of the heme-Fe(III). This observation could explain a lower response obtained with LOD/HRP/FcH-modified CPE when L-lactate solution containing ascorbate was injected (Fig. 3), but it could not support the appearance of anodic peaks with the decrease of the L-lactate/ascorbate ratio. The use of FcH in large excess in the LOD/HRP/FcH-modified CPE in order to minimise the contribution of the reactions between ascorbate and compound-I and/or compound-II was found not sufficient to prevent the interference effect.

In order to understand such electrochemical behaviour, a comparative study was achieved on CPE modified with HRP in the presence and absence of ascorbate, but without adding H_2O_2. In this case, we can assume that the concentrations of both compound-I and compound-II are negligible. So, the regeneration of native HRP from compound-I and compound-II by ascorbate could be supposed as null.

The voltammogram performed without ascorbate in solution (Fig. 5) showed typical electrochemical behaviour of HRP confined into the carbon paste. The addition of ascorbate to the buffered solution caused a change in the CV shape (Fig. 7). The oxidation of ascorbate appeared at the oxidation potential of the heme-Fe(II) showing a catalytic oxidation behaviour. The electrocatalytic peak intensity increased with the ascorbate concentration. Notice that voltammograms performed in the presence of higher ascorbate amount (Fig. 7) showed not only the first peak at a potential close to −40 mV vs. Ag/AgCl, which likely squared with the electrocatalytic oxidation of ascorbate by HRP but showed also a second smaller peak around the potential of +250 mV vs. Ag/AgCl, which could be related to the direct electrochemical oxidation of ascorbate at the electrode surface [39]. At this point, it can be postulated that the heme-Fe(III)/heme-Fe(II) redox couple of HRP could present an electrochemical catalytic behaviour towards ascorbate. Therefore, the electrochemical behaviour exhibited with HRP-modified CPEs in the presence of ascorbate could be supported by an electrocatalytic scheme

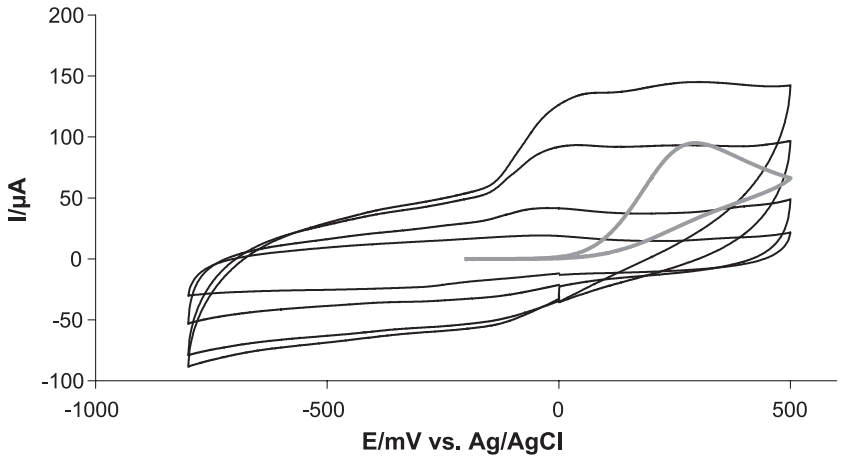

Fig. 7. Cyclic voltammogram of HRP-modified carbon paste electrode in pH 7.2 deoxygenated phosphate buffer (0.1 M containing 0.1 M KCl) at room temperature for increasing amounts of ascorbate (0, 1, 5 and 10 mM). Scan rate 50 mV s^{-1}. The grey curve refers to the cyclic voltammogram shown by a bare CPE in the same experimental conditions in the presence of a 5 mM solution of ascorbate.

similar to the one already proposed in the case of Cyt-*C* [40]:

$$\text{HRP-Fe(III)} + \text{AA} \rightarrow \text{HRP-Fe(II)} + \text{P}$$

$$\text{HRP-Fe(II)} \rightarrow \text{HRP-Fe(III)} + e^- \text{ (at electrode)}$$

where AA is ascorbate and P is the oxidation product. The authors themselves pointed in the case of Cyt-*C* that the heme-Fe(III)/heme-Fe(II) redox couple was involved in the electrocatalytic oxidation of ascorbate.

Regarding literature, we noted that, with the native HRP in solution, the formal redox potential of the heme(Fe^{III})/heme(Fe^{II}) generally is around -265 mV vs. NHE (-482 mV vs. Ag/AgCl) [41]. According to this value, one can conclude that the oxidation of AA ($E^{\circ\prime}_{DAA/AA} \cong -136 \pm 10$ mV vs. Ag/AgCl) by heme-(Fe^{III}) of the native HRP would not be thermodynamically allowed. Hence, how can the electrocatalytic oxidation of AA observed on the HRP-modified electrode be explained?

In this study, HRP was adsorbed on graphite and our results clearly displayed a significant change in electrochemical properties of HRP with an estimated $E^{\circ\prime}$ of heme(Fe^{III})/heme(Fe^{II}) couple found in the order of -170 ± 40 mV vs. Ag/AgCl. This value is significantly different from the one measured for the native HRP in solution but remains similar to those reported in the literature for various HRP-modified electrodes (-100 mV [32], -210 mV [35], -300 mV [31], all values given vs. Ag/AgCl). On the whole, a significant effect on the formal potential was observed when HRP was adsorbed or immobilised on the electrochemical transducer. This could be due to the presence of oxygenated groups (–OH and =O) as phenolic or quinone on the graphite surface that induce some electrostatic interactions with hemic structure and affect the value of formal potential. Another explanation could also relate $E^{\circ\prime}$ variation to the influence of the conformation/orientation of adsorbed HRP on the electrochemical properties of hemic group. Thus, on this basis, we can assume that the determined formal potential (-170 ± 40 mV vs. Ag/AgCl) could represent the average value corresponding to the HRP adsorbed on various orientations.

Then, we note that in this study both E_{pa} and E_{pc} were determined with the precision of -35 ± 10 and -275 ± 50 vs. Ag/AgCl, respectively. Higher variability was observed in the determination of E_{pc} probably due to the presence of oxygen trace remaining into the carbon paste [42]. In fact, the electrocatalytic behaviour of HRP towards the reduction of oxygen could plausibly cause a slight shift of E_{pc}. By taking into account this observation, we can assume that the real value of the formal potential of heme(Fe^{III})/heme(Fe^{II}) ($E^{\circ\prime} \cong -170 \pm 40$ mV vs. Ag/AgCl) should be less negative. All these suggestions indicate that $E^{\circ\prime}$ of HRP could be similar or slightly higher than $E^{\circ\prime}$ of AA (-136 ± 10 mV vs. Ag/AgCl). From a thermodynamic point of view, this would make possible the oxidation of ascorbate by HRP explaining the electrochemical behaviour observed in Fig. 7.

3.3. Contribution of ascorbate on L-lactate amperometric response

We demonstrated in the beginning of this article in Fig. 3 that the effect of ascorbate on the L-lactate measurement was found significant even for an L-lactate/ascorbate ratio of 1:1. A decrease in the signal of 10% was observed when lactate solution (40 µM) was injected in the presence of 40 µM of ascorbate. The first and obvious explanation for these results could be supported by the fact that the ascorbate compete with FcH to regenerate the active form of HRP via reduction of both compound-I and compound-II. However, it is also important to point out that in our

study, we demonstrated that the electrochemical effect of ascorbate of ascorbate observed on the HRP-modified CPE occurred independently from the presence and absence of both FcH and H_2O_2. The use of FcH in a large excess in the LOD/HRP/FcH-modified CPE did not influence the electrochemical contribution of ascorbate. To follow this analysis, we can assume that neither compound-I nor compound-II would be involved in the oxidation of ascorbate on the surface of the HRP-modified CPE.

According to the electrocatalytic scheme proposed above, one can suggest that the native form of HRP-Fe(III) could be reduced in an heme-Fe(II) species in the presence of ascorbate in the solution and regenerated at the electrode surface leading to an anodic current. Concurrently, in the case of the determination of L-lactate on a LOD/HRP/FcH-modified CPE, the electroenzymatic detection of L-lactate via H_2O_2 involved the oxidation of this same native form heme-Fe(III) to compound-II. Therefore, the amperometric signal obtained after injecting the solution of L-lactate in the presence of higher levels of ascorbate could result from both anodic and cathodic processes involving the contribution of heme-Fe(III). Cyclic voltammograms showed in Fig. 7 support this assumption and might explain the ascorbate contribution on the L-lactate response reported in Fig. 3.

From a biosensor development point of view, it is now relevant that the accurate determination of L-lactate requires the minimisation of ascorbate contribution in the electrochemical process involving HRP. Thus, the influence of HRP loading in CPE on the electrocatalytic current of ascorbate oxidation was first examined in the absence of L-lactate using hydrodynamic voltammograms. In order to make easier the comparison, all peaks were normalised with respect to the highest signal of ascorbate solution

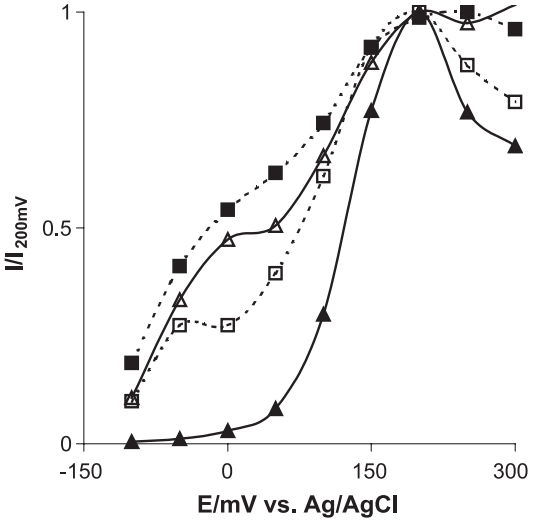

Fig. 8. Hydrodynamic voltammograms of ascorbate (200 μM) on FcH (▲) and LOD/HRP_i/FcH-modified carbon paste electrodes. HRP_i loadings in carbon paste: HRP_1 (■): 13.6 $U.mg^{-1}$, HRP_2 (△): 9.2 $U.mg^{-1}$, HRP_3 (□): 4.7 $U.mg^{-1}$. Flow rate: 0.7 ml min^{-1}. Phosphate buffer 0.1 M (0.1 M KCl), pH 7.2.

Table 1
Recovery rate (%) of a 40 μM lactate solution in the presence of ascorbate [FIA mode, LOD/HRP_i/FcH-modified CPE at − 50 mV vs. Ag/AgCl]

	Ascorbate concentration			
	0 μM	40 μM	80 μM	120 μM
HRP_1 (13.6 U mg^{-1})	100	70 ± 1	41 ± 1	32 ± 3
HRP_3 (4.7 U mg^{-1})	100	84 ± 3	73 ± 1	60 ± 3

All peaks were normalised with respect to the signal of the L-lactate solution prepared without ascorbate.

($I_{200\ mV}$). Fig. 8 shows a typical potential-current response of different HRP-modified CPEs to ascorbate solution over the potential range from − 100 to + 300 mV vs. Ag/AgCl. A small influence of HRP loading in CPE on ascorbate signal was obtained for potentials ≤ − 50 mV vs. Ag/AgCl. Beyond this value, the electrochemical signal showed significant dependency on HRP loading in CPE. The electrode prepared with LOD/FcH only did not show significant effect of ascorbate. Clearly, the decrease in HRP loading tends to decrease significantly the electrochemical contribution of ascorbate.

Taking into account these results, the accuracy of the measurements of the L-lactate concentration in the presence of ascorbate could be improved by decreasing HRP loading in CPE. Therefore, further determination of L-lactate was achieved by using LOD/HRP/FcH electrodes containing different HRP loadings. The resulting measurements were then compared to those obtained by using UV kit. The decrease in the HRP loading in the carbon paste led to slight but significant improving in the accuracy (Table 1). As expected, the recovery rate increased when HRP loading in the CPE decreased showing lower interferences.

4. Conclusion

Despite a low value of operating potential, the accuracy of the LOD/HRP/FcH biosensor was found significantly affected by the presence of ascorbate for the determination of L-lactate. The accuracy of the biosensor was found dependent on the L-lactate/ascorbate ratio. A significant anodic current was obtained even at potentials between − 150 and 0 V vs. Ag/AgCl. The oxidation of ascorbate appeared at the potential of the heme-Fe(III)/heme-Fe(II) redox couple of HRP. The Cyt-*C* type mechanism was suggested to explain the electrocatalytic behaviour of HRP towards the oxidation of ascorbate. These results were useful to better understand the negative effect of ascorbate on the accuracy of the L-lactate electrode.

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