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ORIGINAL PAPER

# Direct electrochemistry of chemically modified catalase immobilized on an oxidatively activated glassy carbon electrode

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**Abstract** Catalase (Ct) was modified using Woodward's reagent K (WRK) as a specific modifier of carboxyl residues. The modified Ct was immobilized on an oxidatively activated glassy carbon electrode surface to investigate its direct electrochemistry. Using cyclic voltammetry an irreversible reduction peak was obtained at approximately −0.362 V vs. Ag/AgCl in buffer solution, pH 7, and at a scan rate of 0.1 V $s^{-1}$. The electrochemical parameters, including charge-transfer coefficient (0.27), apparent heterogeneous electron transfer rate constant (13.51 ± 0.42 $s^{-1}$) and formal potential of the Ct film (−0.275 V) were determined. The prepared enzyme electrode exhibited a response to $H_2O_2$.

**Keywords** Catalase · Direct electrochemistry · Chemical modification · Woodward's reagent K · Hydrogen peroxide

## 1 Introduction

Direct electron transfer between redox proteins and electrode surfaces has attracted considerable attention in the past decade [1]. It can provide a working model to understand the electron transfer mechanism in biological systems and to establish a foundation for fabrication of electrochemical biosensors and bio-electro-analytical devices that do not use any mediators [2–4]. To obtain and improve direct electrochemistry of redox proteins an approach is to immobilize them on electrode surfaces. The protein immobilization methods can be of either physical or chemical nature, or of a mixed type: (a) cross linking with bi-functional reagents; (b) incorporation in polymeric films; (c) entrapment in polymeric gel lattice and microcapsules; (d) covalent binding to polymeric or porous carriers with the aid of activation reagents [5–9].

The incorporation of proteins in polymeric films is one of the most common methods used in investigation of direct electrochemistry of redox proteins on carbon electrode surfaces [10–17]. However, the films are not efficient for large proteins since their electroactive prosthetic groups are deepened within the protein structure. As a result, it is difficult for these proteins to directly exchange electrons with the electrode surface. Also adsorptive denaturation of the proteins may occur on the electrode surface [2, 18, 19]. It is, therefore, pertinent to increase the electron transfer rate and catalytic activity by exploring and developing new ways of protein preparation on electrode surfaces.

Binding of proteins, as nucleophiles, to polymeric carboxyl carriers via activation reagents like carbodiimide and Woodward's reagent K (WRK) is considered as an effective method to investigate electrochemistry of proteins. In this method, carboxyl groups of the carriers will be converted to active enol esters, which can subsequently react with nucleophilic groups on proteins to affect on the immobilization of the macromolecules [20–23]. The WRK also was used to modify the carboxyl residues of some proteins including catalase (Ct) into active enol esters. The activated carboxyl groups are sufficiently stable to allow isolation of the modified protein; easily determination of the extent of modification; and in a subsequent step, the

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isolated modified protein can be reacted with nucleophilic reagents. Such a separation of the activation step from nucleophilic displacement allows one to choose conditions which are optimal for each step. This situation is not possible in the procedure with water-soluble carbodiimide, since the activation and displacement steps must be carried out simultaneously [24–26].

Considering the ability of proteins to react with WRK, we applied this method for chemical modification of catalase as a large redox enzyme. Although the direct electron transfer of catalase using various modified electrodes has already been reported [12, 13, 27–32], to our knowledge the direct electrochemistry of chemically modified catalase immobilized on oxidatively activated glassy carbon electrode (GCE) has not been reported yet. Using this approach the direct electrochemistry of Ct and its catalytic activity toward hydrogen peroxide was investigated.

## 2 Experimental

### 2.1 Reagents

Bovine liver Ct (EC 1.11.1.6) and 2-ethyl-5-phenylisoxazolium-3-sulfonate (Woodward's reagent K) were purchased from Sigma-Aldrich. Perfluorinated ion-exchange resin (Nafion, 5% in ethanol) was obtained from Aldrich and hydrogen peroxide (30%) was purchased from Merck.

### 2.2 Chemical modification of catalase

Crystalline Ct was dissolved in 50 mM phosphate buffer (PB) pH 7.0 and sufficiently dialyzed against buffer. The concentration of Ct stock solution was determined by measurement of its absorbance at 405 nm, using $3.24 \times 10^5$ $M^{-1}$ $cm^{-1}$ for the molar extinction coefficient [33] and 250,000 Daltons for the molecular mass of the enzyme. Selective modification of exposed carboxylate groups of Ct (0.5 mg $mL^{-1}$) was carried out with WRK (10 mM) using the procedure described in our previous work [24].

### 2.3 Apparatus and measurements

A Shimadzu UV-3100 spectrophotometer equipped with a water bath (± 0.5 °C) was used for determination of catalase concentration as well as for recording spectra of native and WRK-modified Ct in the wavelength ranges 200–700 nm.

A PARC 263A potentiostat/galvanostat (EG&G, USA) was used for voltammetric measurements. A conventional three-electrode cell was used with an Ag/AgCl electrode, a platinum rod and a GCE (diameter 0.2 mm) as reference, counter and working electrode respectively. Prior to voltammetry, solutions were purged with purified nitrogen for at least 15 min and a nitrogen atmosphere was maintained over the solutions during experiments.

Scanning electron microscopy (SEM) images of the immobilized films on GCE were obtained with a ZIESS EM 902A scanning electron microscope. For each SEM image, the electrode tip was detached from the electrode body and coated with a thin layer of gold.

### 2.4 The working electrode preparation

Before each experiment, the working electrode was cleaned in the following manner. It was polished with 0.06 μm alumina suspension and then on a filter paper. Thereafter, it was rinsed with deionized water and sonicated with a Tecno-Gaz ultrasonic cleaner (Italy) in a beaker containing deionized water for 2 min. The electrode was oxidatively activated in 50 mM PB pH 7 for 2 min. at an applied potential of +1.5 V. Then the electrode surface was coated with 10 μL (0.5 mg $mL^{-1}$) of chemically modified Ct (WRK–Ct). Afterwards, 2-μL of 5% Nafion was spread onto the enzyme-coated GCE (WRK–Ct–Nafion). Finally, it was allowed to dry at room temperature for 3 h. A similar coating, but without the enzyme, was prepared to be used as control experiments. Nafion is a perfluorosulfonate ionomer that contains less than 15% ionizable sulfonate groups per monomer unit and has hydrophobic perfluoro-poly-ether chains which can bind to hydrophobic moiety of macromolecules. Incorporating the macromolecules into Nafion film, is revealed to improve their permeability and stability [34–36].

## 3 Results and discussion

### 3.1 Absorption spectra

The UV–Vis absorbance spectra of the native Ct and WRK–Ct are shown in Fig. 1. As seen, modification of the enzyme by WRK results in the appearance of a new absorption peak in the wavelength range 340–350 nm, implying the modification of aspartate or glutamate residues and the formation of a stable enol–ester bond [24].

### 3.2 Morphological characterization of the WRK–Ct–Nafion coated electrode

The surface morphology of the film coated electrode was characterized by SEM. As seen in Fig. 2a, the deposition of WRK–Ct–Nafion generated a non-uniform membrane

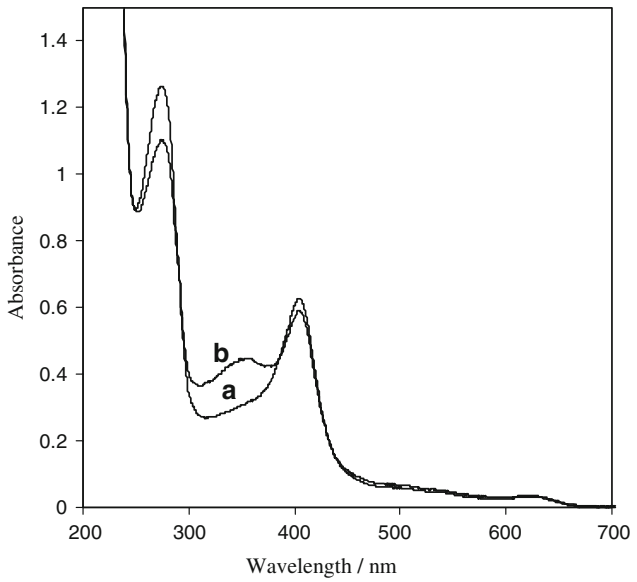


**Fig. 1** UV–Vis absorbance spectra of the native (a) and modified catalase (b). Catalase was modified by 10 mM of WRK. The protein concentration was 0.5 mg/mL at pH 7.0

where dark spots of protein emerge over a polymer coating. At higher magnification the WRK–Ct–Nafion demonstrates a complex crystal structure (Fig. 2b). Figure 2c, shows the structure of Nafion film. It is generally obvious that Nafion has a micro-phase separated morphology of spherical shaped hydrophilic regions containing sulfonate groups, embedded in hydrophobic regions composed by a perfluorinated back bone [37]. Therefore, it seems the high molecular weight catalase is covered by the polymeric net. Most certainly, the WRK–Ct–Nafion film on GCE produces a high heterogenic membrane, where the polymer has deformed microstructure and essentially acts as a protein binding agent.

### 3.3 Electrochemistry of WRK–Ct–Nafion film

The electrochemical behavior of the WRK–Ct–Nafion film on a GCE in 50 mM PB solution, pH 7.0, was studied by cyclic voltammetry. The cyclic voltammograms (CVs) overlaid in Fig. 3 show the redox behaviors of Ct–Nafion film (curve b) and WRK–Ct–Nafion film (curve c) on the GCE at scan rate of 0.1 V $s^{-1}$. The CV acquired from the Nafion immobilized GCE in the absence of Ct is also given (curve a). The Ct–Nafion film and the Nafion immobilized on the GCE exhibits no essential voltammetric response, while an irreversible reduction peak is observed at about −0.362 V vs. Ag/AgCl for the WRK–Ct–Nafion film. It is noteworthy that the WRK–Ct (without Nafion film) on the GCE exhibited a weak and wide irreversible reduction peak which was obscured rapidly with soaking time (data not shown). In another experiment, the behavior of Ct, WRK and WRK–Ct in PB solution, pH 7.0, were examined independently. They also showed no voltammetric responses (data not shown). Thus, it seems that chemical

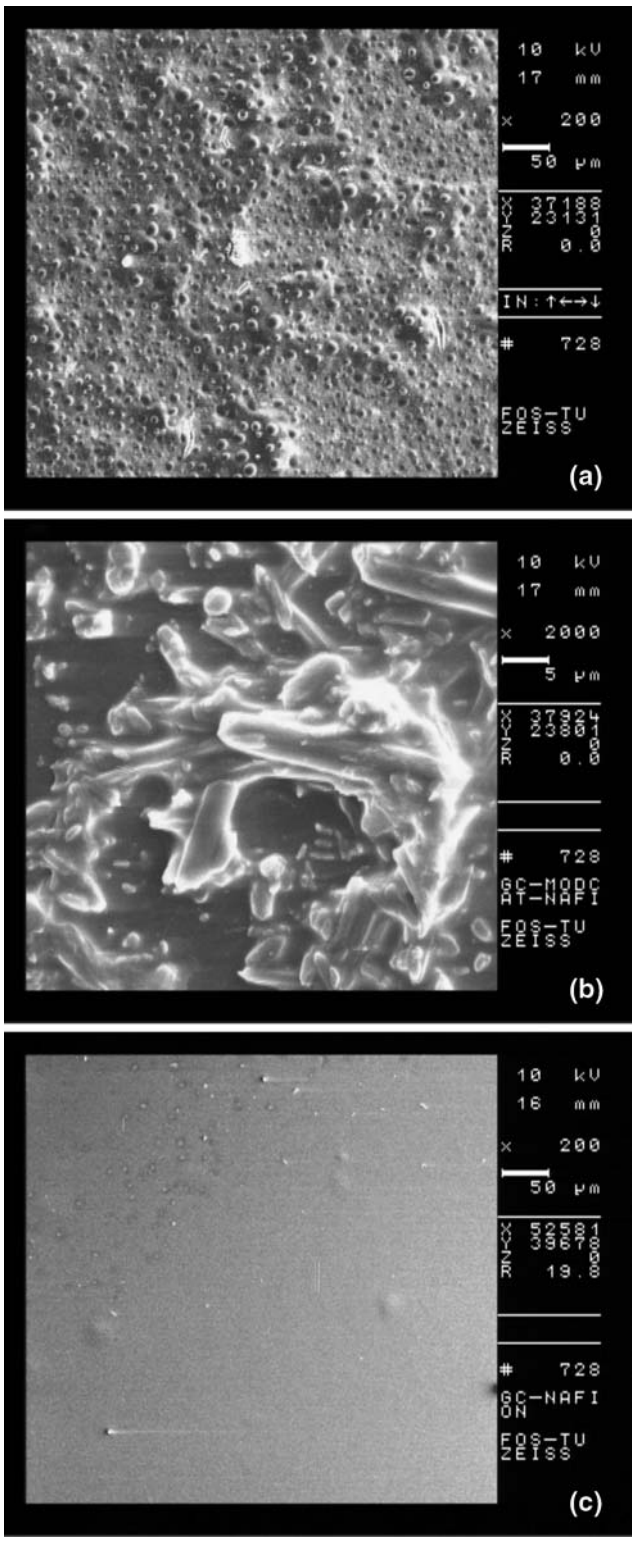


**Fig. 2** SEM images of electrode surfaces covered by: (**a**) WRK–Ct–Nafion film, (**b**) WRK–Ct film, (**c**) Nafion film

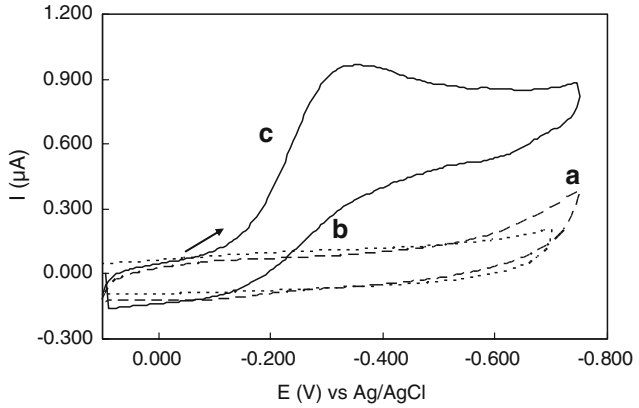


**Fig. 3** Cyclic voltammograms of (a) Nafion film, (b) Ct–Nafion film, (c) WRK–Ct–Nafion film on glassy carbon electrode. The experiments were carried out at scan rate 0.1 V $s^{-1}$ in 50 mM phosphate buffer pH 7. The arrow indicates the scan direction

modification along with immobilization of the enzyme provides a favorable condition for the enzyme to exchange electrons with the underlying GCE. The cathodic peak potential ($E_{pc}$) is close to the characteristic potential of the Ct–Fe(III) reduction (−0.399 V) [27].

To obtain the kinetic parameters of the immobilized enzyme on the activated GCE, the scan rate effect was investigated (Fig. 4a). A linear dependence of cathodic peak current versus scan rate ranging from 0.050 to 0.800 V $s^{-1}$ is observed (Fig. 4b), indicating the electrochemical behavior of WRK–Ct–Nafion–GCE. The amount of electroactive enzyme on the electrode surface can be estimated from the slope of peak currents plotted versus scan rate. For irreversible reduction of an adsorbed species the slope is given by the Eq. 1 [38]:

$$\text{slope} = \frac{\alpha n^2 F^2 A \Gamma}{2.718 RT} \tag{1}$$

where, Γ (mol $cm^{-2}$) is the amount of adsorbed WRK–Ct, α is the charge-transfer coefficient, A ($cm^2$) is the electrode surface area, and the other symbols have their usual meanings. Assuming a single electron transfer reaction, the amount of electroactive protein molecules is estimated to be $6.5 \times 10^{-11}$ mol $cm^{-2}$. This value is only 2.6% of the total amount of WRK–Ct deposited on the electrode surface. It is noteworthy that the relative amount of electroactive proteins on the electrode surface is always low for all heme proteins [13, 16, 27]. This may suggest that only those proteins situated in the inner layers of the films close to the electrode and with a suitable orientation can exchange electrons with the electrode surfaces.

The kinetic parameters of α and heterogeneous transfer rate constant ($k_s$) is estimated using the Laviron model (for $n\Delta E_p > 0.200$ V, Eq. 2) [39].

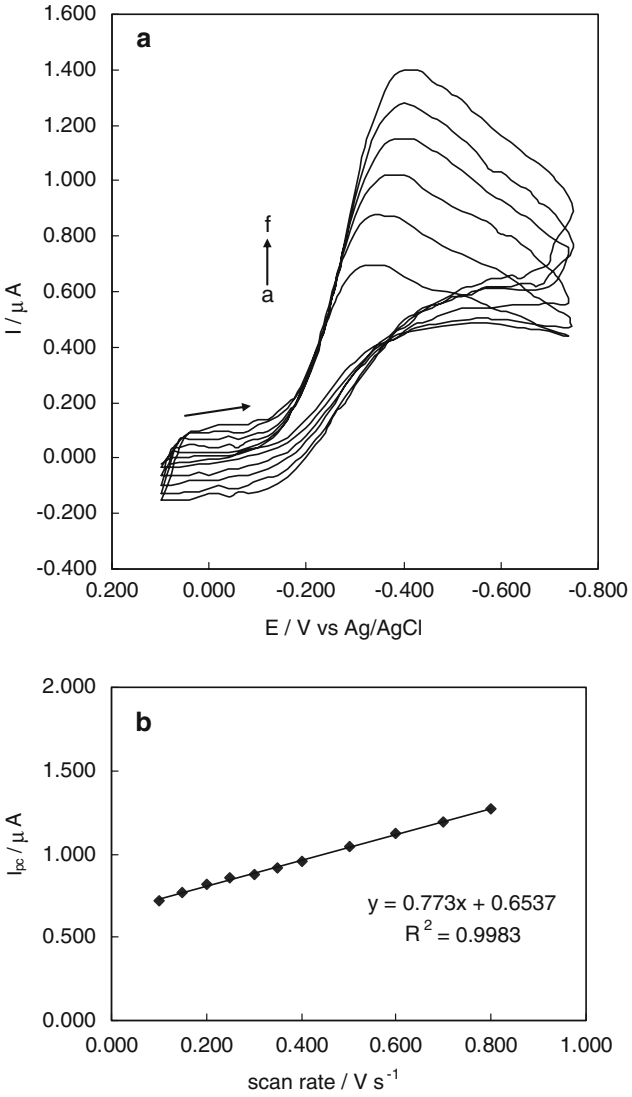


**Fig. 4** (**a**) Cyclic voltammograms of WRK–Ct–Nafion film at the glassy carbon electrode in 50 mM phosphate buffer solution (pH 7) over a range of scan rates (a–f: 0.050, 0.100,.0.2000.300, 0.400, and 0.500 V $s^{-1}$). (**b**) Cathodic current versus the scan rate. The arrow indicates the scan direction

$$\log k_s = \alpha \log(1-\alpha) + (1-\alpha)\log\alpha - \log\frac{RT}{nFv} - \frac{\alpha(1-\alpha)nF\Delta E_p}{2.3RT} \tag{2}$$

Here, $v$ is the scan rate, $\Delta E_p = |E_p - E'^0|$ is the difference between peak potential ($E_p$) and formal potential ($E'^0$). The other symbols have their usual meanings. The $E'^0$ can be determined by extrapolating $E_{pc}$ plotted versus scan rate [39]. The $E_{pc} - v$ plot exhibits a linear relationship ($R^2 = 0.9929$) with an intercept of −0.275 V as $E'^0$ (plot not shown). A graph of the peak potential versus the logarithm of the scan rate yields a straight line at scan rates >0.200 V $s^{-1}$ (Fig. 5). From the slope of this line (−2.3RT/nFα), a value of charge transfer coefficient was

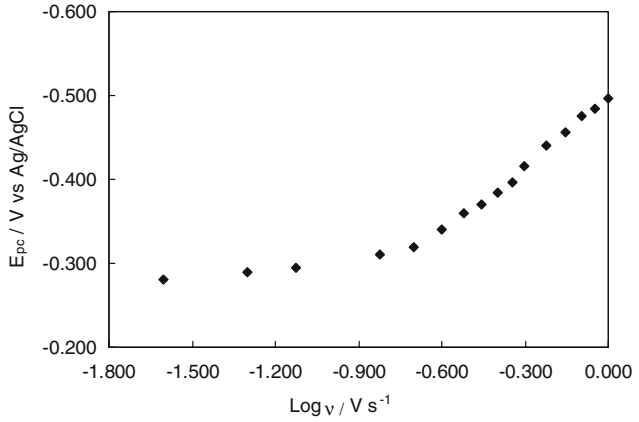


**Fig. 5** Influence of scan rate on cathodic peak potential for a WRK–Ct–Nafion film on the glassy carbon electrode in 50 mM phosphate buffer (pH 7.0)

calculated ($\alpha = 0.27$). An average $k_s$ value was then obtained as $13.51 \pm 0.42\ s^{-1}$.

The apparent heterogenous electron transfer rate constant, the cathodic peak potential and the charge transfer coefficient for WRK–Ct–Nafion film and Ct-agarose film [27] are compared in Table 1. The higher $k_s$ value for WRK–Ct–Nafion film indicates that this immobilization method provides a favorable microenvironment for catalase and enhances the rate of electron transfer between the enzyme and electrode.

### 3.4 Reproducibility and stability of the WRK–Ct–Nafion film electrode

The reproducibility of the WRK–Ct–Nafion electrode response described above was examined at five electrodes prepared under the same conditions. The relative standard deviation (% R.S.D.) was 9.6% for an average $i_{pc}$ of 789 nA at scan rate 0.1 V $s^{-1}$ in 50 mM phosphate buffer pH 7.

The stability of WRK–Ct–Nafion film electrode was investigated by cycling the electrode potential over the range +0.1 to −0.6 V. According to Fig. 6, after 45 cycles the peak potential remained nearly unchanged while the peak current reduced 16%. At this point, the peak currents showed no more decrease even after 24 h. The decrease in current is probably due to the loss of loosely attached WRK–Ct from the electrode surface.

**Table 1** Electron transfer kinetic parameters for catalase

| Films | $E_{pc/V}$ | $K_s/s^{-1}$ | α | Ref. |
|---|---|---|---|---|
| WRK–Ct–Nafion | −0.362 | 13.51 ± 0.42 | 0.27 | Present work |
| Ct–agarose | −0.399 | 1.0 ± 0.1 | 0.82 | 27 |

Apparent heterogenous electron transfer rate constant ($k_s$), cathodic peak potential ($E_{pc}$) and charge transfer coefficient (α) for catalase films on GCE at pH 7.0 and scan rate 100 mV/s

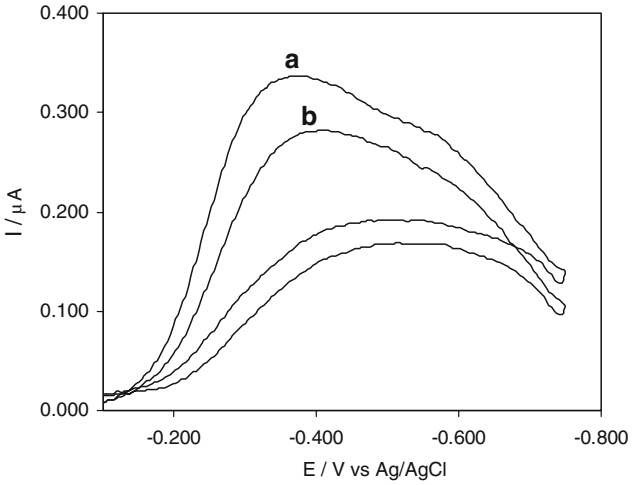


**Fig. 6** The 1st (**a**) and 45th (**b**) recorded cyclic voltammogram of WRK–Ct–Nafion film on GCE in 50 mM phosphate buffer solution (pH 7) at scan rate 25 mV $s^{-1}$

### 3.5 Influence of pH on voltammetry

An increase of buffer pH revealed a negative shift in the $E_{pc}$ of the Ct–Fe(III) reduction (Fig. 7A). This shift in the peak potential of WRK–Ct–Nafion may result from protonation/deprotonation of a water molecules at the sixth coordination position in the heme iron and also from protolytic groups around the heme while pH was changed [40]. The $E_{pc}$ of the chemically modified enzyme was linearly proportional to pH value in the range of 4.5–9.0 (Fig. 7B) with linear regression equation of $E_{pc} = -0.032$ pH–0.098 ($R^2 = 0.992$), indicating that the redox reaction is accompanied by proton transfer. The value of the slope, 32 mV $pH^{-1}$, is smaller than the theoretically expected value of 59 mV $pH^{-1}$ for the reaction of one electron coupled one proton [41]. This might be due to the influence of the protonation states of trans ligands of the heme iron and amino acids around the heme, or the protonation of the water molecule coordinated to the central iron [42].

### 3.6 Catalytic activity of WRK–Ct

Bio-electrocatalytic reduction of hydrogen peroxide was carried out on WRK–Ct–Nafion GCE by linear sweep voltammetry in 50 mM PB solution (pH 7.0) and at scan rate of 0.100 V $s^{-1}$ (Fig. 8A). By increasing the concentration of $H_2O_2$, an increase in the reduction peak is observed while; in the applied potential range and in the absence of enzyme, $H_2O_2$ was not reduced on either bare GCE or Nafion/GCE. Figure 8B shows catalytic efficiency, expressed as a ratio of the reduction peak current of WRK–Ct–Nafion films in the presence ($I_c$) and absence ($I_d$) of $H_2O_2$. By increasing the concentration of $H_2O_2$ up to about 35 μM, the catalytic efficiency is increased, and then it

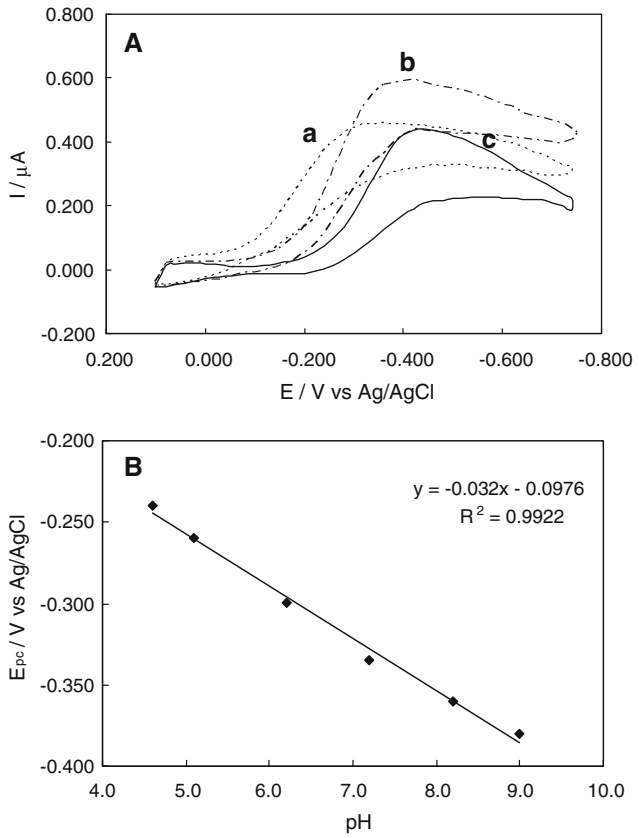


**Fig. 7** (**A**) Cyclic voltammograms of WRK–Ct–Nafion film on the GCE in 50 mM phosphate buffer (a) pH 4.6, (b) pH 7.2 and (c) pH 9.0. Scan rate was 0.100 V $s^{-1}$. (**B**) Cathodic peak potential ($E_{pc}$) versus pH

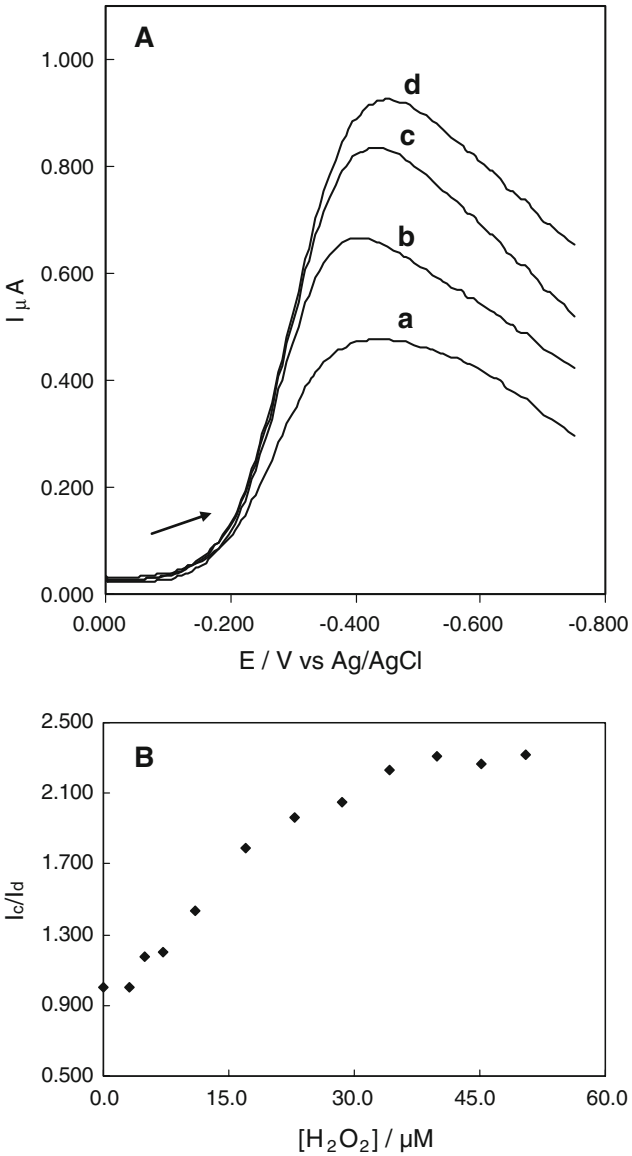


**Fig. 8** (**A**) Linear sweep voltammograms of WRK–Ct–Nafion film at the glassy carbon electrode in the absence (a) and presence of 11 µM (b), 23 µM (c) and 34 µM (d) $H_2O_2$. (**B**) Catalytic efficiency changes versus the concentration of $H_2O_2$, where $I_c$ and $I_d$ are the cathodic peak currents in the presence and absence of $H_2O_2$, respectively. The electrolyte solution was 50 mM phosphate buffer solution (pH 7.0) and scan rate was 0.1 V $s^{-1}$. The arrow indicates the scan direction

tends to level off. This indicates saturation of the modified enzyme at higher concentrations of $H_2O_2$. The electrode reaction is characteristic of an EC′ mechanism [38, 43]:

$$\text{WRK}-\text{Ct}-\text{Fe(III)} + \text{e} + \text{H}^+ \rightarrow \text{WRK}-\text{Ct}-\text{Fe(II)H}$$

$$\text{WRK}-\text{Ct}-\text{Fe(II)H} + 1/2\ \text{H}_2\text{O}_2 \rightarrow \text{WRK}-\text{Ct}-\text{Fe(III)} + \text{H}_2\text{O}$$

where, WRK–Ct–Fe(III) and WRK–Ct–Fe(II)H denote the oxidized and reduced forms of the modified enzyme, respectively. Initially WRK–Ct–Fe(III) undergoes the electron transfer reaction with the electrode and resulting in the production of WRK–Ct–Fe(II)H, which in turn, can be oxidized by $H_2O_2$ in the solution to regenerate WRK–Ct–Fe(III). In this catalytic reaction, the peak current keep rising until WRK–Ct–Fe(III), consumed in the first reaction is compensated by its production in the second reaction. In other words, an irreversible electron transfer was followed by an irreversible regeneration of starting material by $H_2O_2$. The above electrode reaction slightly differs from the conventional EC′ mechanism due to the fact that both WRK–Ct–Fe(III) and WRK–Ct–Fe(II)H are surface-confined.

### 3.7 Reaction mechanism for the immobilization of Ct on GCE

Based on our finding, Ct can react with WRK as a specific modifier for surface carboxyl residues (Fig. 1) [24]. That is because, Ct with high acidic residues, mostly found on the out side of the molecule, is considered as an acidic protein [44, 45]. As presented in Fig. 9, the reaction of WRK with carboxylate groups proceeds rapidly to form enol esters [25, 26]. This reaction is initiated by the abstraction of a proton from the reagent by a base or water, causing an

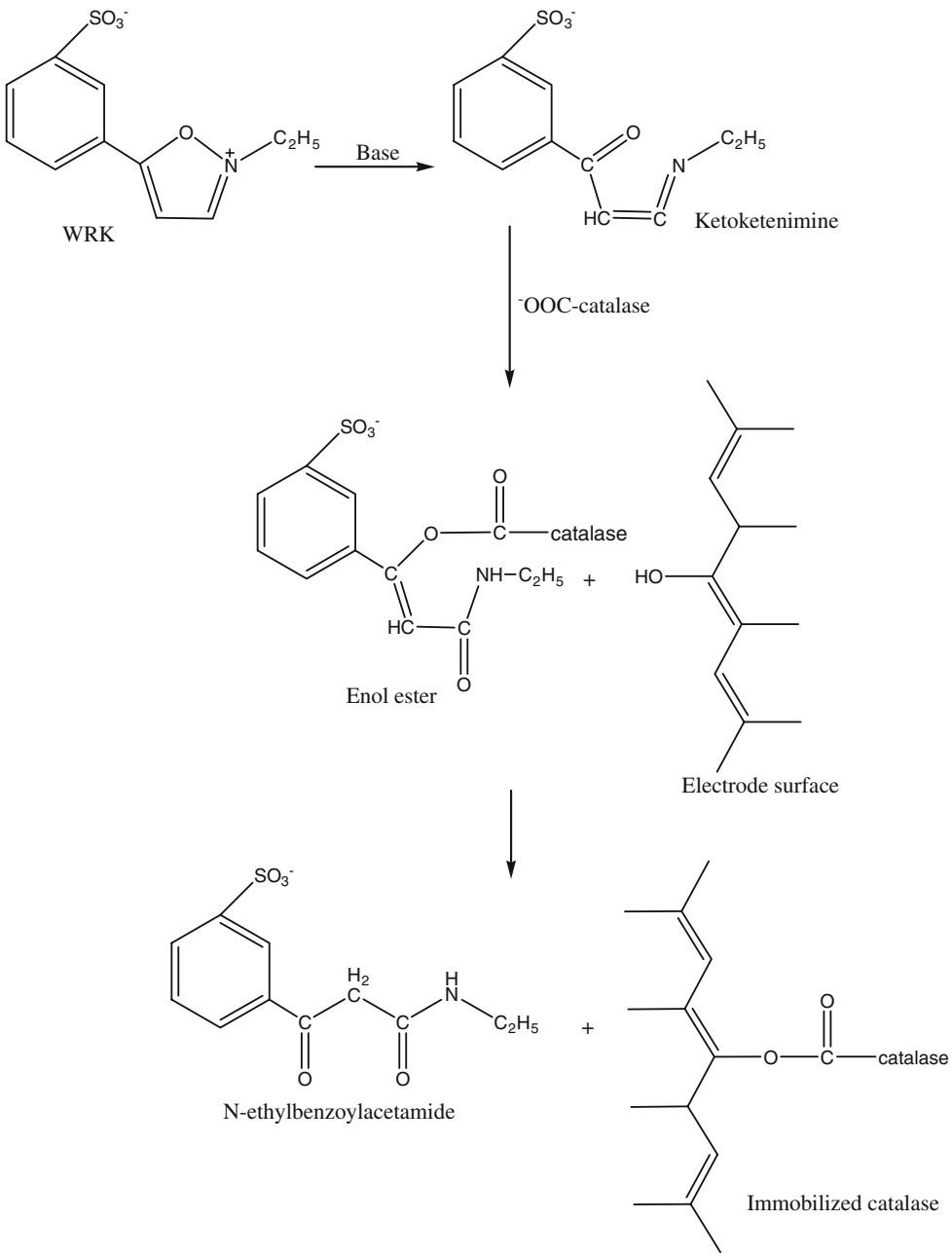


**Fig. 9** Reaction mechanism of catalase with WRK and the proposed mechanism for the immobilization of catalase on the activated glassy carbon electrode

immediate rearrangement to highly reactive ketoketenimine intermediate, which then rapidly reacts with a carboxylate group on the protein to form an enol ester. The formed enol esters may further react with appropriate nucleophiles [25, 46]. Here we used an electrochemically activated GCE as the partner that participate in direct electrochemistry of Ct. During the oxidative treatment, oxidized functional groups appear, which are mainly alcoholic or phenolic in nature [47, 48]. These functional groups can act as nucleophiles, which may react with enol ester derivative of Ct resulting in an immobilized enzyme.

It is difficult for large proteins such as Ct to directly exchange electrons with the electrode surface because the adsorptive denaturation of these proteins may occur on electrode surface [2, 18, 19]. Our previous results revealed that during chemical modification of Ct with WRK, the secondary structure of the modified enzyme did not change significantly, while the fluorescence properties of 1-anilino-8-naphthalenesulfonate (ANS) interacting with WRK–Ct showed a sharp quenching of ANS fluorescence, which was indicative of a decrease in exposed hydrophobic patches on the modified enzyme [24]. Furthermore, the oxidative treatment of the electrode was shown to cause a decrease in hydrophobic groups on the electrode [47]. Based on this evidence it seems that the modification of Ct, along with the oxidative treatment of the electrode, inhibits

the adsorptive denaturation of the protein via decreasing hydrophobic interactions between the enzyme and electrode surface. As a result formation of the enol ester derivative of catalase makes it possible to show direct electron transfer and catalytic activity at the electrode surface.

## 4 Conclusion

WRK–Ct–Nafion film on GCE was shown to be able to establish a direct electron transfer between the enzyme and electrode and to exhibit electrocatalytic activity toward hydrogen peroxide. It seems that the modification of Ct along with the oxidative treatment of the electrode inhibits the adsorptive denaturation of the protein via decreasing hydrophobic interactions between the enzyme and electrode surface. Therefore, in comparing the different methods that have been applied to investigate direct electrochemistry of redox proteins [3, 49], the method used in the present report is unique from the aspect that the protein modifier does not show electrochemical behavior, but just provides favorable conditions for electron transfer between the electrode surface and enzyme active sites.

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