J Appl Electrochem (2009) 39:971–977
DOI 10.1007/s10800-009-9776-7

ORIGINAL PAPER

Development of a new biosensor for mediatorless voltammetric determination of hydrogen peroxide and its application in milk samples

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Received: 7 April 2008 / Accepted: 29 October 2008 / Published online: 16 January 2009

Abstract A new biosensor for the voltammetric detection of hydrogen peroxide was developed based on immobilization of catalase on a clinoptilolite modified carbon paste electrode using bovine serum albumin and glutaraldehyde. The biosensor response was evaluated according to electrode composition, reaction time, solution pH and temperature. The voltammetric signals were linearly in proportion to H_2O_2 concentration in the range 5.0×10^{-6}–1.0×10^{-3} M with a correlation coefficient of 0.9975. The detection limit is 8.0×10^{-7} M and the relative standard deviation for 4.0×10^{-4} M hydrogen peroxide was 1.83% ($n = 6$). The biosensor exhibited high sensitivity, and it was determined that it could be used for more than 2 months. In addition, the biosensor was successfully applied for the determination of hydrogen peroxide in milk samples.

Keywords Hydrogen peroxide · Voltammetry · Biosensor · Clinoptilolite · Catalase · Milk

1 Introduction

Hydrogen peroxide is a product of reactions catalyzed by oxidases or catalase and is important in many areas including biochemistry, food chemistry, clinical chemistry and environmental chemistry. The determination of hydrogen peroxide is of great importance for the food industry because it is used as an antibacterial agent for milk, and it has to be removed by catalase before milk turns microbiologically into cheese. Many selective and sensitive determination methods for hydrogen peroxide have been developed using titrimetry, spectrophotometry, and electrochemistry [1–5]. However, conventional methods for the determination of hydrogen peroxide do not offer high sensitivity, reliability and operational simplicity at the same time, and they also have complex reactions. Among these methods, amperometric sensors based on electron transfer between an enzyme and on electrode are promising in preparing sensitive and linearly responding devices. Although a direct electron transfer is possible between an electrode and an enzyme catalyzing the reduction of hydrogen peroxide, mediators are generally used to accelerate the reaction rate [6]. Various biosensors based on adsorption, cross-linking self assembly and incorporation into carbon paste, conducting polymer and different kinds of gels such as hydro gels and sol-gels have been developed for the determination of hydrogen peroxide [7–14]. All the methods mentioned above had achieved great success; however they also suffered from short-comings. A disadvantage of the direct adsorption of peroxides on the electrode surface is that the biosensor has a short lifetime due to desorption of the enzyme from the electrode surface [13]. Many zeolites are well suited to co-immobilize enzyme and mediator for biosensor preparation as their large porous surface area and hydrophilic surface are favourable for mediator loading on the zeolites and decrease the resistance to material transportation of the substrate. This method acquired a fast response and high stability to the sensor [15].

In this study, clinoptilolite, a natural zeolite, was used as an immobilization matrix for the first time to incorporate

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catalase in carbon paste without any electron transfer mediator. The aim of the study is to improve the analytical performance of biosensor by means of clinoptilolite, to demonstrate the unnecessariness of mediator usage, and to develop a relatively inexpensive biosensor. Differential pulse and cyclic voltammetric measurements were employed to show the use of the developed biosensor for determination of hydrogen peroxide. The applicability of the developed biosensor for determination of hydrogen peroxide in milk samples was also demonstrated.

2 Experimental

2.1 Chemicals and materials

Clinoptilolite, a natural zeolite, was supplied by Enli Mining Corporation (Bornova-Izmir, Turkey) from the Manisa Gordes Region, Turkey. Graphite fine powder (GP) (Aldrich) and mineral oil (Sigma) were used for the carbon paste electrode (CPE). Hydrogen peroxide, H_2O_2, (30% w/w) was purchased from Merck, and the concentration of dilute hydrogen peroxide prepared from this material was determined by titration with potassium permanganate. Catalase (EC: 1.11.1.6), bovine serum albumin (BSA) and glutaraldehyde were obtained from Sigma. Syringe micropore membrane filters were purchased from Sigma-Aldrich (0.45 μm). Phosphate buffer solutions (PBS) were prepared by mixing the KH_2PO_4 and K_2HPO_4, and the pH was adjusted with either H_3PO_4 or NaOH. Glycine buffer was also used for experiments. All solutions were prepared daily with deoxygenated high purity water from a USF ELGA UHQ water purification system (18 MΩ cm^{-1}).

2.2 Apparatus

All differential pulse voltammetry (DPV) and cyclic voltammetry (CV) experiments were performed using a Metrohm 746 trace analyser and 747 VA stand. Conventional three electrode systems were employed with the modified carbon paste as working electrode, a Ag/AgCl (sat.KCl) as reference electrode and a platinum wire as auxiliary electrode. The electrochemical experiments were performed in a voltammetric cell with 10 mL solutions. Before the measurements all solutions were purged with high purity nitrogen. All experiments were carried out at 35 °C under a nitrogen atmosphere.

2.3 Preparation of the carbon paste biosensor

Carbon paste electrodes (CPE) were made by mixing 0.70 g graphite powder and 0.30 g of mineral oil. Carbon paste electrodes modified by catalase (CAT) and clinoptilolite (Cli) were prepared by mixing 50.0 mg graphite powder, 50.0 mg clinoptilolite, 3.0 mg catalase (2,350 U/mg) and 40.5 mg of mineral oil. In addition, carbon paste electrodes containing both graphite (100.0 mg) and catalase (3.0 mg) (CAT-CPE), and containing graphite (53.0 mg) and clinoptilolite (50.0 mg) (Cli-CPE) were also prepared. CPE and modified CPE were packed firmly into a cylindrical tube (3.3 mm inner diameter and 5 cm length), respectively, and then the electrode surface was smoothed with clean weighing paper. Electrical contact was established via copper wire inserted into the CPE. CATCli-CPE was obtained after covering the electrode surface with 0.1% (w/v) BSA and 2.5% (v/v) glutaraldehyde. Finally, the CATCli-CPE surface was rinsed with distilled water. After each experiment, the CATCli-CPE was stored at 4 °C.

2.4 Preparation of milk samples

The hydrogen peroxide concentrations in ultra-high-temperature (UHT) processed milk samples were voltammetrically determined by the standard addition method. Voltammetric analyses were conducted with diluted milk samples. Firstly, 2.5 mL of milk sample was added to 22.5 mL of 0.1 M phosphate buffer solution (pH 7.0). Secondly, 2.5 mL of milk sample and 2 mL of 1.0×10^{-2} M H_2O_2 were added to 20.5 mL of 0.1 M PBS (pH 7.0). In order to compare results, milk samples were also analyzed by titration with potassium permanganate. Before the titration, 2.5 mL of 3 M H_2SO_4 was added to 2.5 mL of milk sample and centrifuged for 10 min and then sample was filtered with syringe micropore membrane filters (0.45 μm).

2.5 Procedure

Experiments were carried out in a voltammetric cell containing 10 mL of 0.1 M PBS at pH 7.0. The PBS was purged with pure nitrogen for 10 min before all the voltammetric measurements. Hydrogen peroxide solution was prepared in 0.1 M phosphate buffer solution (pH 7.0) de-aerated for 30 min and then it was transferred to the voltammetric cell. Catalase catalyses the decomposition of H_2O_2, and this led to production of O_2 and H_2O. Hence, the differential pulse voltammetry was conducted for the reduction of O_2. Reactions which occur at the biosensor surface are given as a schematic diagram in Fig. 1. The DPV was performed at a scan rate of 15 mV s^{-1} with a pulse amplitude of 50 mV, a pulse time of 40 ms and a measuring time of 20 ms. The differential pulse voltammograms were obtained in the cathodic direction between 500 and −1,000 mV.

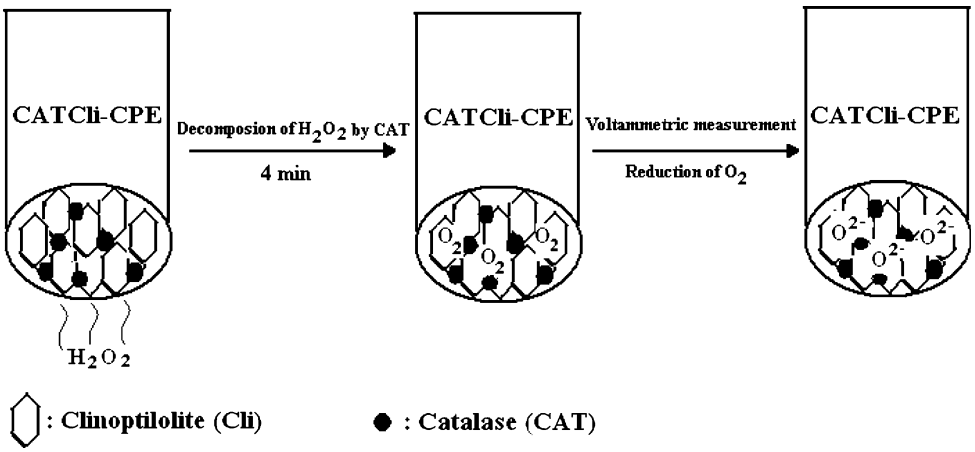

Fig. 1 Schematic diagram of the reactions at the CATCli-CPE surface

3 Results and discussion

3.1 Cyclic voltammetric behaviour of the CATCli-CPE

The cyclic voltammetric behaviour of hydrogen peroxide was studied in 0.1 M phosphate buffer (pH 7.0) with different electrodes such as CPE, CAT-CPE, Cli-CPE, and CATCli-CPE, as shown in Fig. 2a–d. However, no electrochemical signal was observed in the absence of H_2O_2 with all electrodes, but an obvious current was observed at −480 mV for all electrodes in the presence of H_2O_2. Comparison of the voltammograms for the H_2O_2 signal of different electrodes shows that the sharpest and the highest peak current was observed at the CATCli-CPE. It was also observed that the electrode surface was enriched by means of casting O_2 molecules in pores and channels of clinoptilolite since H_2O_2, well catalyzed the CATCli-CPE, by the formation and inclusion O_2 molecules. As a result, increasing oxygen concentration on the CATCli-CPE surface caused a higher peak current.

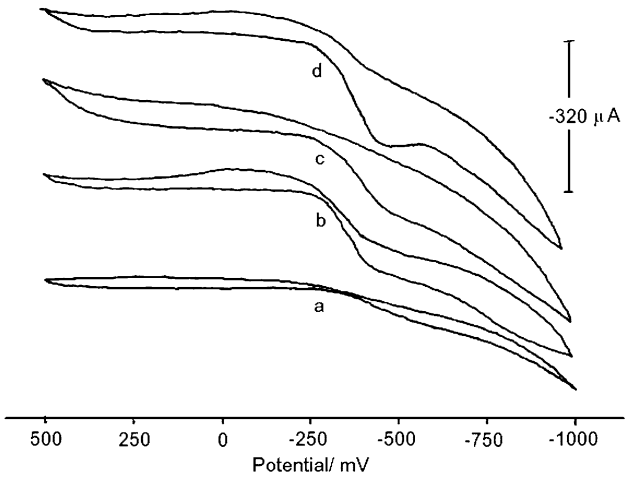

Fig. 2 Cyclic voltammograms of (**a**) CPE, (**b**) CAT-CPE, (**c**) Cli-CPE, and (**d**) CATCli-CPE (concentration of H_2O_2: 1.0×10^{-2} M; supporting electrolyte: 0.1 M phosphate buffer at pH 7.0; T: 35 °C; scan rate: 15 mV s^{-1}; reaction time: 4 min)

3.2 Optimization of the carbon paste composition

The amount of clinoptilolite dispersed in carbon paste was changed in the range 10 to 40% w/w. According to the differential pulse voltammograms, negligible changes in the peak current were observed between 0 and 20% w/w, but increasing clinoptilolite content between 20 and 35% w/w led to a significant increase in the peak current. The advantages of clinoptilolite dispersion in carbon paste for biosensor response are demonstrated in Fig. 3a–b. As the clinoptilolite amount in the carbon paste increases the peak current increases. No desirable electrochemical signal was found at more than 40% w/w clinoptilolite containing CPE because the electrode surface could easily disperse during the experiments, and after decrease in percentage of graphite in CPE, a low conductive electrode, led to an increase in background current and a decline in sensitivity of hydrogen peroxide determination.

To investigate the effect of enzyme amount on biosensor response, carbon paste electrodes were prepared at different ratios. Figure 4a–b depicts the dependence of the biosensor peak current on the enzyme amount in the carbon paste. It was observed that the electrode response increased with an increasing amount of enzyme between 1 and 3 mg and remained stable at higher values. Hence, 3 mg enzyme was chosen for all subsequent preparations of the carbon paste biosensor.

3.3 The effect of solution pH on the CATCli-CPE response

The effect of pH on the CATCli-CPE response was studied in the pH range 2.0–10.0 using phosphate and glycine

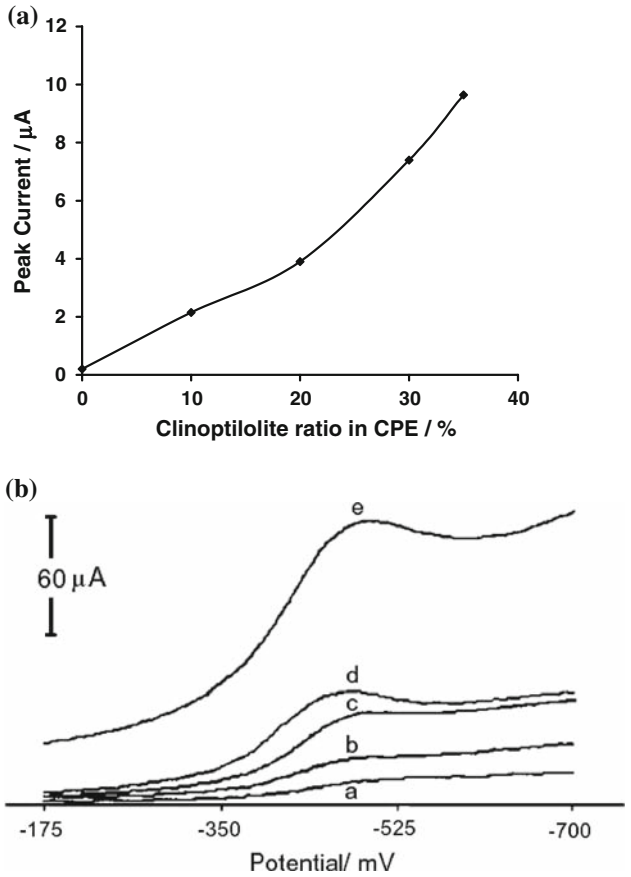

Fig. 3 **a** A plot for the effect of clinoptilolite ratio changing between 0% (w/w) and 35% (w/w) in the CAT-CPE on the electrode response. **b** Voltammograms a: 0%; b: 10%; c: 20%; d: 30%; and e: 35% (w/w) (concentration of H_2O_2: 4.0×10^{-4} M; 3 mg catalase in CPE; supporting electrolyte: 0.1 M phosphate buffer at pH 7.0; T: 35 °C; scan rate: 15 mV s^{-1}; reaction time: 4 min)

buffers in the presence of 4.0×10^{-4} M H_2O_2. As shown in Fig. 5, the peak current increased from pH 4.0 to 7.0, and the maximum peak current was obtained at pH 7.0. At greater than pH 7.0 the peak current gradually decreased. In order to obtain maximum sensitivity and bioactivity, phosphate buffer solution at pH 7.0 was selected as a working buffer.

3.4 The effect of temperature on the CATCli-CPE response

Figure 6 shows the effect of temperature on the CATCli-CPE response in the range between 20 and 50 °C for 4.0×10^{-4} M H_2O_2. The results indicated that the peak current increases with temperature up to 35 °C. Further increase of temperature gives rise to a decrease of the peak current because of partial denaturation of the enzyme. Thus, the experimental temperature was controlled at 35 °C.

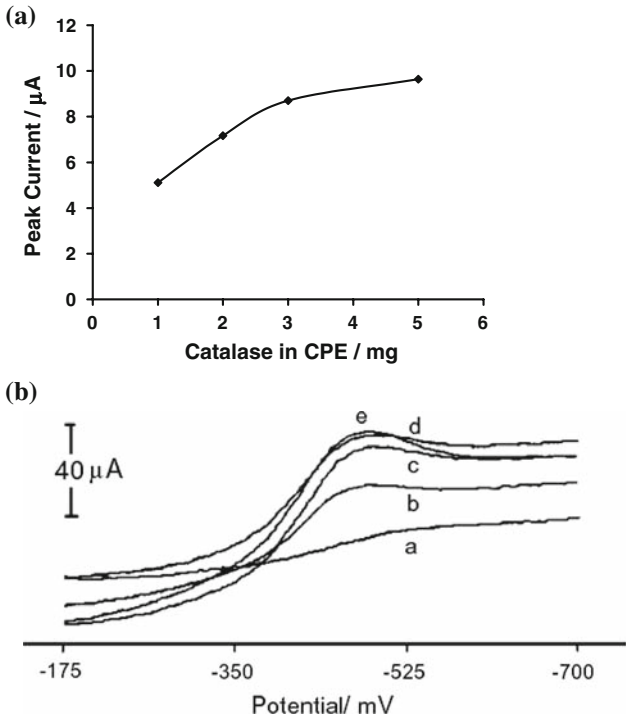

Fig. 4 **a** A plot for the effect of enzyme amount changing between 1 and 5 mg in the Cli-CPE on the electrode response. **b** Voltammograms a: background current; b: 1; c: 2; d: 3; and e: 5 mg (concentration of H_2O_2: 4.0×10^{-4} M; supporting electrolyte: 0.1 M phosphate buffer at pH 7.0; T: 35 °C; scan rate: 15 mV s^{-1}; reaction time: 4 min)

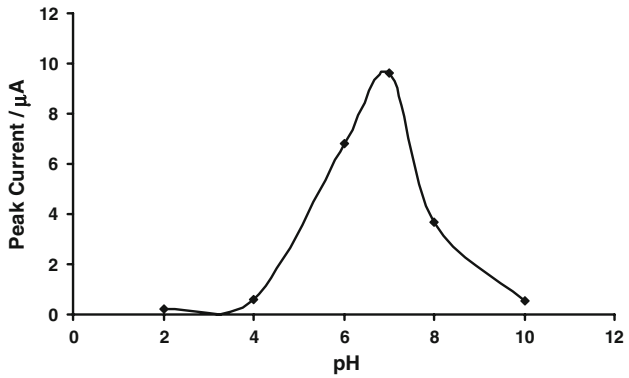

Fig. 5 The effect of pH on the biosensor response (concentration of H_2O_2: 4.0×10^{-4} M; biosensor composition: 50.0 mg GP/50.0 mg Cli/3.0 mg CAT; supporting electrolyte: 0.1 M phosphate buffer; T: 35 °C; scan rate: 15 mV s^{-1}; reaction time: 4 min)

3.5 Analytical characteristics of the CATCli-CPE

3.5.1 Linear range and detection limit of the CATCli-CPE

At optimum experimental conditions, the CATCli-CPE response was linear for hydrogen peroxide within the concentration range 5.0×10^{-6}–1.0×10^{-3} M ($r = 0.9975$) as depicted in Fig. 7. The CATCli-CPE provides a wider

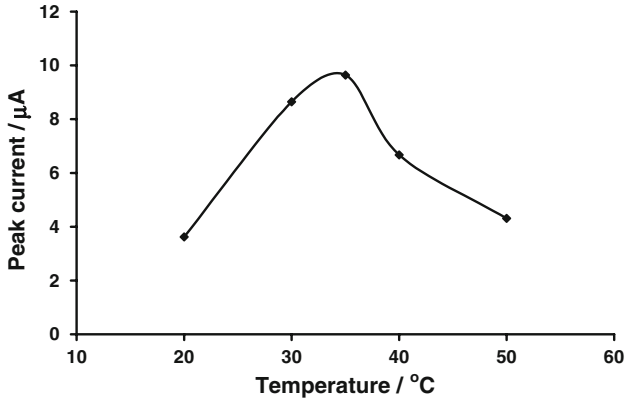

Fig. 6 The effect of temperature on the biosensor response (concentration of H_2O_2: 4.0×10^{-4} M; biosensor composition: 50.0 mg GP/50.0 mg Cli/3.0 mg CAT; supporting electrolyte: 0.1 M phosphate buffer at pH 7.0; scan rate: 15 mV s^{-1}; reaction time: 4 min)

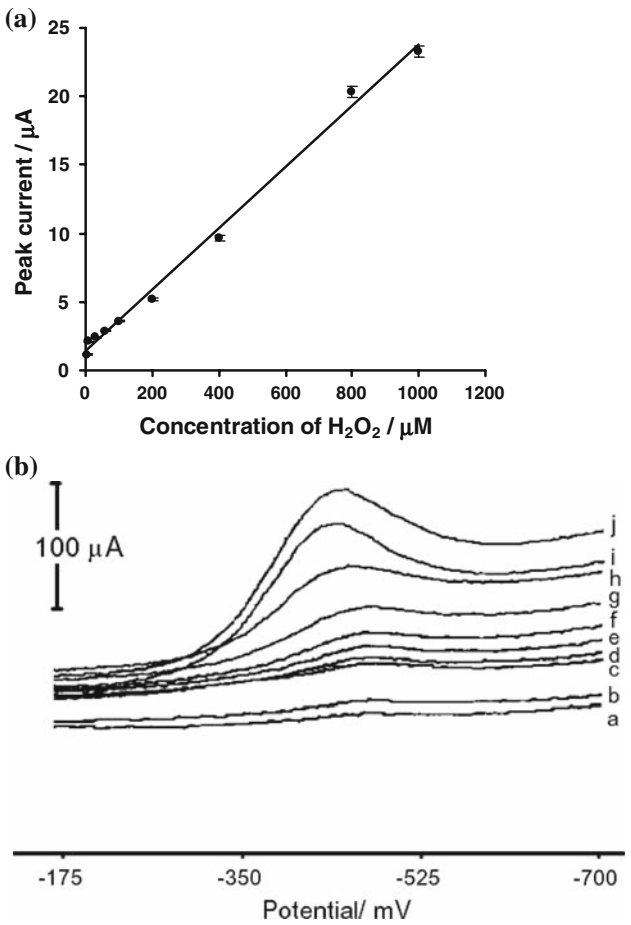

Fig. 7 **a** A calibration curve of the biosensor. **b** Voltammograms a: background current; b: 5.0×10^{-6}; c: 1.0×10^{-5}; d: 3.0×10^{-5}; e: 6.0×10^{-5}; f: 1.0×10^{-4}; g: 2.0×10^{-4}; h: 4.0×10^{-4}; i: 8.0×10^{-4}; and j: 1.0×10^{-3} M H_2O_2 (biosensor composition: 50.0 mg GP/50.0 mg Cli/3.0 mg CAT; supporting electrolyte: 0.1 M phosphate buffer at pH 7.0; T: 35 °C; scan rate: 15 mV s^{-1}; reaction time: 4 min). R.S.D.% values for each point of the calibration curve are between 1.77 and 1.90 ($n = 5$)

linear range of detection than those of reference systems based on the electrodes without enzymes such as modified carbon paste electrodes, modified glassy carbon electrode, glassy carbon electrode modified by cytochrome c/NaY zeolite, gold electrode modified by DNA-Hb, modified Pt electrode, and than those of reference systems based on the biosensors modified by horseradish peroxidase (HRP) such as immobilization of mediator in carbon paste, sol-gel/hydrogel composite film, silicon oxide, sol-gel/chitosan composite film, carbon nanotubes/chitosan composite film, lipid membrane, and pyrolytic graphite [13, 14, 16–26]. When compared to other biosensors modified by catalase, and to spectrofluorometric methods, the CATCli-CPE again has a wider linear range [27–29]. The CATCli-CPE has a detection limit of 8.0×10^{-7} M with an estimated signal-to-noise ratio of 3. The CATCli-CPE was more sensitive than biosensors modified by HRP, modified carbon paste electrodes, modified glassy carbon electrode, modified disk, gold and ITO electrode and biosensor modified by catalase [16, 17, 23, 24, 26, 30–37]. When we compare the CATCli-CPE with glassy carbon electrodes modified by HRP/zeolite/mediator, glassy carbon electrodes modified by HRP/sol gel, and Pt electrode modified by HRP/polypyrrole, the CATCli-CPE has a similar sensitivity [13, 15, 38, 39].

3.5.2 *Reproducibility*

The electrode to electrode reproducibility was studied using the response to 4.0×10^{-4} M H_2O_2. The response of CATCli-CPE was found to be very reproducible with a relative standard deviation (R.S.D.%) of 1.83% observed for the response of six freshly prepared biosensors. This electrode-to electrode reproducibility value is better than gold electrodes modified by HRP, ITO electrode modified by HRP/Au, ITO electrode modified by DNA/Hb/Au, sol-gel/hydrogel composite film modified by HRP, Pt electrode modified by HRP/polypyrrole and glassy carbon electrode modified by HRP [13, 30, 31, 37, 39–41].

3.5.3 *Storage stability*

The long-time stability of the CATCli-CPE was investigated over an 8-week period to detect the decreases in the biosensor response. The storage stability of the biosensor was examined periodically every week. The biosensor was kept at 4 °C in a flask containing distilled water when not used. All measurements were conducted in 0.1 M phosphate buffer (pH 7.0) at 35 °C. After the storage period, the remaining activity of the biosensor was measured. The stability of the biosensor was acceptable, as the biosensor maintained 96.2% of its initial activity at the end of 4 weeks and 78.8% at the end of 8 weeks as demonstrated in Fig. 8.

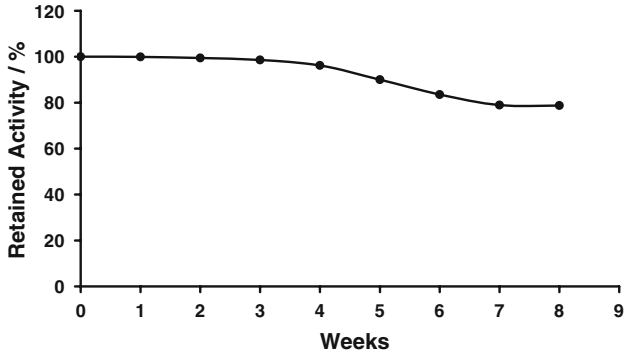

Fig. 8 Storage stability of the biosensor (biosensor composition: 50.0 mg GP/50.0 mg Cli/3.0 mg CAT; supporting electrolyte: 0.1 M phosphate buffer at pH 7.0; T: 35 °C; scan rate: 15 mV s^{-1}; reaction time: 4 min)

Table 1 Hydrogen peroxide concentration in milk samples

Milk samples	Concentration of hydrogen peroxide (w/v %)	
	Measured by CATCli-CPE	Determined by titrimetric method
1	0.0121 ± 0.075	0.0120 ± 0.264
2	0.0085 ± 0.083	0.0083 ± 0.352

Results are expressed as mean ± S.D., $n = 3$

3.5.4 Real sample analysis

The application of the CATCli-CPE was evaluated by the determination of hydrogen peroxide concentration in real samples. Due to the fact that hydrogen peroxide is often used as a preservative agent in milk, milk samples were chosen in order to demonstrate the application of the CATCli-CPE to real samples. The concentrations of hydrogen peroxide in milk samples were determined with the standard addition method. Voltammetric results were compared with those determined by the classical potassium permanganate titration method, and given in Table 1. The results determined by the CATCli-CPE were in satisfactory agreement with those given by the titration method.

4 Conclusion

A combination of clinoptilolite and catalase, which has a very high turnover number, have been used for the first time to determine hydrogen peroxide concentration. Oxygen produced after the catalysis reaction between catalase and hydrogen peroxide, was cast in the channels or pores of the clinoptilolite structure. The experiment of the voltammetric detection of hydrogen peroxide was conducted by the reduction of produced oxygen. The use of clinoptiolite led to an improvement of the biosensor stability and sensitivity. For the determination of hydrogen peroxide, compared with the existing zeolite modified electrodes, glassy carbon electrode modified by cytochrome c/NaY zeolite and glassy carbon electrodes modified by HRP/zeolite/mediator, the developed biosensor has some advantages in view of simplicity, low cost, unnecessariness of mediator and its wider linear range [15, 19, 38]. The existing zeolite modified electrodes were developed by using synthetic zeolite combining with electron transfer mediators, cytochrome c, methylene blue and methylene green, and detection of the hydrogen peroxide was conducted amperometrically. The CATCli-CPE biosensor differs from existing zeolite modified sensors in terms of both the preparation method of the biosensor and the detection procedure of the hydrogen peroxide. In this work, the biosensor was developed employing a low cost natural zeolite and catalase without using any mediator. The developed biosensor was also successfully applied to the determination of hydrogen peroxide in real samples. Considering its stability, sensitivity, simplicity, fast response and low cost, the biosensor modified by clinoptilolite and catalase can be considered an attractive the method for the determination of hydrogen peroxide.

Acknowledgments The authors express their thankfulness to Associate Prof. Dr. Stephen T. Astley, Department of Chemistry, Faculty of Science, Ege University, for his careful review of the manuscript.

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