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Analytical Methods

Determination of calcium in milk and water samples by using catalase enzyme electrode

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ABSTRACT

A biosensor based on catalase enzyme was developed for the investigation of the effect of calcium ions on the activity of the enzyme. Calcium plays an activator role for the catalase enzyme that catalyses the degradation of hydrogen peroxide to O_2 and H_2O. Determination method of the effect of calcium ion on the activity of the enzyme was based on the assay of the differences on the responses of the biosensor in the absence and the presence of calcium in the reaction medium. The biosensor had a linear relation to calcium concentrations and good measurement correlation between 1 and 10 mM with 1 min response time. Tris–HCl buffer (pH 7.0; 50 mM) and 37 °C were obtained as the optimum working conditions. In the application studies, the biosensor was used determination of calcium level of real samples such as milk, spring and mineral water.

1. Introduction

Calcium (Ca) is the most abundant mineral in the human body. It is important for intracellular metabolism, bone growth, blood clotting, nerve conduction, muscle contraction and cardiac functions (Cáceres, García, & Selgas, 2006). Calcium and calcium salts are also very important minerals used in the food industry. Different calcium salts have been studied for decay prevention, sanitation and nutritional enrichment of fresh fruits and vegetables. Calcium carbonate and calcium citrate are the main calcium salts added to foods in order to enhance the nutritional value. Calcium lactate, calcium propionate and calcium gluconate have shown some of the benefits of the use of calcium chloride, such as product firmness improvement, and avoid some of the disadvantages (Alzamora et al., 2005). Also, the use of calcium salts other than calcium chloride could avoid the formation of carcinogenic compounds (chloramines and trihalomethanes) linked to the use of chlorine (Manganaris, Vasilakakis, Diamantidis, & Mignani, 2007).

There are many methods for detection of calcium such as biosensors (Mank et al., 2006; Xinyan et al., 2008), continuos flow analysis (CFA) (Traversi et al., 2007), HPLC (Paull, Macka, & Haddad, 1997), electrochemical methods (Bratov, Abramova, Domínguez, & Baldi, 2000; Chen & Adams, 1998; Chumbimuni-Torres & Kubota, 2006; Komaba et al., 1998; Saurina, López-Aviles, Le Moal, & Hernández-Cassou, 2002; Song & Chen, 2003; Wang, Xu, Zhang, & Liu, 2008), spectrophotometric methods (Benamor & Aguerssif, 2008; Chen & Jiang, 2002; Demetrius, Paraskevas, Aristidis, & John, 1999; Libarona & Iñón, 2005), X-ray fluorescence (Alvarez, Marcó, Arroyo, Greaves, & Rivas, 2003; Ekinci, Ekinci, Polat, & Budak, 2005), atomic absorption methods (Bugallo, Segade, & Gómez, 2007; Kmetov, Stefanova, Hristozov, Georgieva, & Canals, 2003; Udoh, 2000), ion-selective electrodes (van Staden & Stefan, 1999) and ion chromatography (Waterworth & Skinner, 1998; Yu, Yuan, Nie, & Yao, 2001).

Catalase, which degrades H_2O_2 into water and oxygen, is one of the major antioxidant enzymes (Scandalios, Guan, & Polidoros, 1997). It is one of the first enzymes to be purified and crystallised and has gained a lot of attention in recent years because of its link to cancer, diabetes and ageing in humans and animals (Melov et al., 2000; Preston, Muller, & Singh, 2001). There are many evidences that the changes of catalase activity as well as the mechanisms of its regulation are essential in the response to stress situations. A very important mechanism in the plant cell participating in regulation of many biochemical pathways is the interaction between the enzyme molecule and different small molecules present in the enzyme surrounding. These small molecules might be products of normal metabolic processes or are normal constituents of plant tissues as metal ions. Ca^{2+} ion is one of them that activated the catalase enzyme (Kocsy, Owttrim, Brander, & Brunold, 1997; Medina, Botella, Quesada, & Valpuesta, 1997; Navari-Izzo, Quartacci, & Sgherri, 1997; Rao, Paliyath, & Ormrod, 1996).

Activation based enzyme electrodes are the new application in the biosensor area and they are based on the activation of the

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enzyme, used in the biosensor construction, especially by a metal ion or biochemical molecule (Akbayirli & Akyilmaz, 2007; Akyilmaz, Baysal, & Dinçkaya, 2007; Akyilmaz and Yorganci, 2008).

In this study, a new biosensor based on the activation of catalase enzyme by calcium ion was developed for the investigation of the effect of calcium ion on the activity of the enzyme. Determination method of the effect of calcium ion on the activity of the enzyme was based on the assay of the differences on the responses of the biosensor in the absence and the presence of calcium in the reaction medium.

2. Experimental

2.1. Chemicals

Catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase) (EC 1.11.1.6.) from bovine liver 1870 U mg^{-1}, $CaCl_2$, NaCl, KCl, $MgSO_4$, $NiCl_2$, $CuSO_4$, $MnCl_2$, KH_2PO_4, K_2HPO_4, calf skin gelatin, glutaraldehyde (25%), Na_2EDTA, Eriochrome Black T and all other chemicals were purchased from Sigma Chemical Co. (USA). All solutions were prepared with double distilled water just before their use.

2.2. Apparatus

In these experiments, a YSI Model 58 digital oxygen meter with 0.01-mg/l dissolved oxygen (DO) concentration sensitivity, YSI 5700 Model DO probes (with YSI 5740 cable) as transducers, standard teflon membranes (YSI, Yellow Springs, OH, USA), Gilson P100 and P1000 automatic pipets (France), Yellow-Line magnetic stirrer (Germany) and Nuve model thermostat (TR) were used.

2.3. Preparation of the biosensor

First of all, a DO probe was covered with a standard teflon membrane using an O-ring and then the membrane which is sensitive for oxygen was pretreated with 0.5% SDS (sodiumdodecylsulphate) in phosphate buffer (50 mM, pH 7.0) to reduce the tension on the membrane surface of the DO probe. After this step, 250 μl of catalase enzyme solution and gelatin were mixed and dissolved at 38 °C for a few minutes. Two-hundred microlitre of the solution was spread over the DO probe membrane surface and allowed to dry at 4 °C for 30 min. At the end of the time, the bioactive layer was treated with glutaraldehyde (2.5%, in phosphate buffer; 50 mM, pH 7.0) for 3 min to form chemical covalent bonds (Schiff bases) between gelatin, enzyme and glutaraldehyde molecules for the immobilisation of the enzyme on the surface of the DO probe.

2.4. Measurements

In the reaction, catalase converts hydrogen peroxide to hydrogen dioxide and carbon dioxide in the presence of oxygen. There is an intermediate surface between the bioactive layer and the teflon membrane of the DO probe and during the enzymatic reaction dissolved oxygen concentration in the intermediate surface decreased relative to the substrate concentration added into the reaction medium. The measurements with the developed biosensor were carried out at steady-state conditions. ΔDO is the differences of the dissolved oxygen concentration when the substrate is not in the reaction medium and after addition of substrate into the reaction medium to obtain a new steady-state DO concentration. It is well known that calcium is a cofactor for catalase and it plays an activator role for the catalase so when the calcium was injected into the reaction medium it increased the activity of the enzyme and in this case the dissolved oxygen concentration changed relative to the calcium concentration added into the reaction medium. The principle of the measurement of the biosensor was based on the determination of these changes in the dissolved oxygen concentration related to calcium concentrations used in the enzymatic reaction. As a result, the differences between the first and the final dissolved oxygen concentrations related to calcium concentrations were detected by the biosensor to obtain a standard curve for the determination of calcium. All the measurements were carried out at 37 °C using a thermostatic reaction cell and oxygen saturated tris–HCl buffer (50 mM, pH 7.0).

3. Results and discussion

3.1. Detection of calcium effect as an activator on the biosensor responses

At the beginning of the study, some experiments were carried out for the determination of the effect of calcium as an activator on the catalase enzyme biosensor. For this purpose firstly, the developed biosensor was used only for hydrogen peroxide detection using standards with concentration between 1 and 10 mM in the absence of calcium and a linear curve was obtained. After that, by using the same hydrogen peroxide standards but in the presence of 5 mM calcium a new standard curve was obtained. Fig. 1 shows the results obtained from the experiments. According to the figure the biosensor responses increased very efficiently in the presence of calcium.

3.2. Optimisation of the bioactive surface of the biosensor

3.2.1. Effect of the enzyme activity on the biosensor response

Different enzyme amounts were used for determination of the effect of the enzyme activity on the biosensor response. For this purpose, three biosensors which contain 0.414, 0.827 and 1.655 U cm^{-2} catalase, were prepared by immobilising with gelatin (5.31 mg cm^{-2}) and glutaraldehyde (2.5%).

From the experiments it can be said that when the biosensor contained 0.827 U cm^{-2} catalase, the most useful calibration curve was obtained. Calcium was detected with a linear range between 1 and 10 mM concentrations by this biosensor. Increase in the catalase activity from 0.827 to 1.655 U cm^{-2} resulted in higher biosensor responses. When the bioactive layer of the biosensor contained 0.414 and 1.655 U cm^{-2} activity of catalase we didn't obtain any suitable standard curve for calcium. In this case, if we

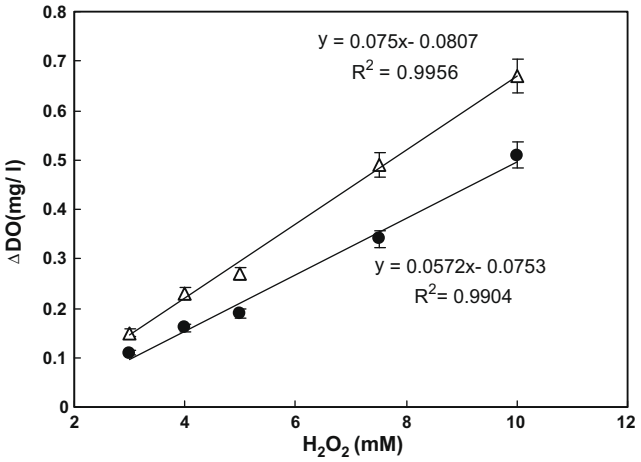

Fig. 1. Detection of the effect of $CaCl_2$ on the activation of catalase enzyme. Tris–HCl buffer; pH 7.0, 50 mM; *T*: 37 °C; (●–●) without calcium; (△–△) with 5 mM $CaCl_2$. The percentage of glutaraldehyde and gelatine amount was kept constant at 2.5% and 5.31 mg cm^{-2}, respectively.

consider the results obtained from the experiments it can be said that the most suitable biosensor responses were obtained by the biosensor which contained 0.827 U cm^{-2} activity of catalase.

3.2.2. Detection of the effect of gelatin amounts on the biosensor response

To determine the effect of the amount of gelatin on the biosensor response different gelatin amounts were used in the construction of the biosensor. Biosensors contained 3.54, 5.31 and 7.08 mg cm^{-2} gelatin and 0.827 U cm^{-2} catalase. All the biosensors were immobilised by 2.5% of glutaraldehyde. The measurements were made in order to obtain standard curves for calcium. The most suitable curve was obtained with the biosensor prepared using 5.31 mg cm^{-2} gelatin amount.

From the experiments when the gelatin amount increased from 5.31 to 7.08 mg cm^{-2}, we obtained higher biosensor responses but there was deviation from linearity. Decreases of the gelatin amounts from 5.31 to 3.54 mg cm^{-2} results in a lower biosensor response. The reason of this effect was the reducing in the forming of cross-linked bonds between enzyme–gelatin–glutaraldehyde. Therefore the enzyme escaped from the bioactive layer. From the results it can be uttered that the most suitable biosensor responses were obtained by the biosensor which contained 5.31 mg cm^{-2} gelatin.

3.2.3. Effect of the percentage of glutaraldehyde on the biosensor response

For the determination of the effect of the percentage of glutaraldehyde on the biosensor response different glutaraldehyde amounts were used in the construction of the biosensor. For this purpose we prepared biosensors which contain 0.827 U cm^{-2} catalase and 5.31 mg cm^{-2} gelatin. The biosensors were treated with 1.25%, 2.5% and 3.75% glutaraldehyde solution prepared in phosphate buffer (50 mM, pH 7.0) for the immobilisation. After the immobilisation procedure, experiments were carried out to obtain standard curves for hydrogen peroxide using the prepared biosensors.

Experiments showed that the higher biosensor responses were observed at 2.5% glutaraldehyde percentage. The biosensors that were prepared with 1.25% and 3.75% glutaraldehyde, showed lower biosensor responses. As a result of the good linear range and high biosensor responses the biosensors were prepared with 2.5% glutaraldehyde.

3.3. Optimisation of working conditions

3.3.1. Effect of pH on the biosensor response

To determine the effect of the pH value on the biosensor response different buffer systems were investigated. For this aim 50 mM concentration of citrate (pH 5.0–6.0), tris–HCl (pH 7.0–8.0) and glycine (pH 9.0–10.0) buffers were used in the experiments. The optimum pH value was determined to be 7.0 (Fig. 2).

From the experiments below and above this pH value decreases in the biosensor responses were observed. If we consider the optimum pH value (pH 7–8) of the free catalase it can be uttered that the immobilisation procedure did not affect the optimum pH value of the enzyme.

3.3.2. Effect of temperature on the biosensor response

The enzyme activity depends on the temperature and the medium conditions. For determination of the effect of temperature on the biosensor response, experiments were carried out between 15 and 40 °C. The highest biosensor response was observed at 37 °C. Below and above this degree, decreases in the biosensor responses that probably resulted from the changes in the enzyme structure, were recorded.

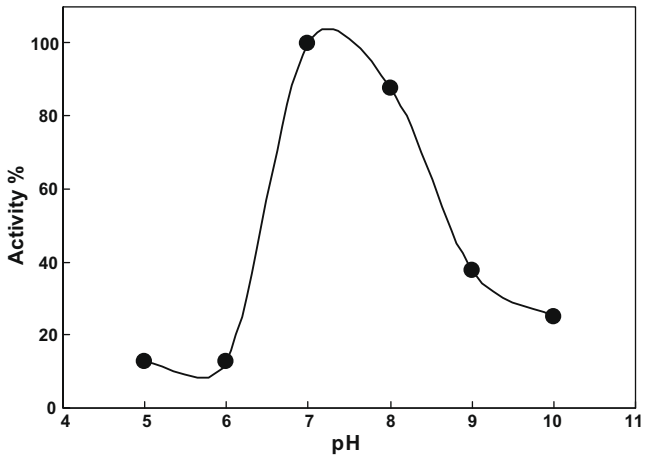

Fig. 2. The effect of pH on the biosensor response (50 mM; citrate buffer (5.0–6.0)), tris–HCl buffer (7.0–8.0) and glycine buffer (9.0–10.0), *T*: 37 °C. The activity of catalase, the percentage of glutaraldehyde, gelatine amount, the concentration of hydrogen peroxide and calcium were kept constant at 0.827 U cm^{-2}, 2.5% and 5.31 mg cm^{-2}, 5 and 5 mM, respectively.

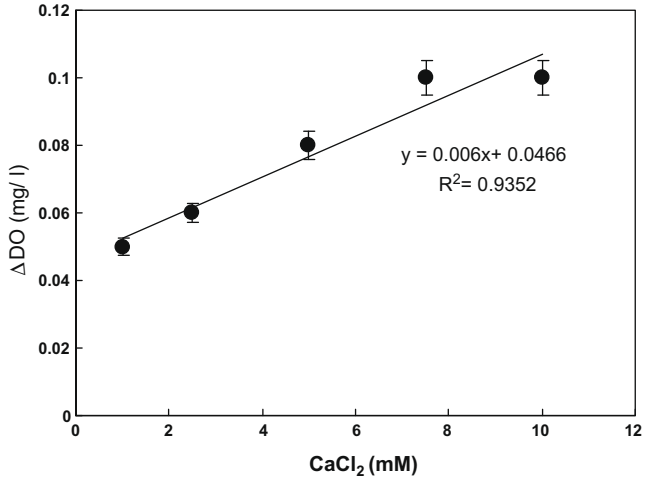

Fig. 3. Standard curve for calcium determination (tris–HCl buffer; pH 7.0, 50 mM; *T*: 37 °C). The activity of catalase, the concentration of hydrogen peroxide, the percentage of glutaraldehyde and amount of gelatin were kept constant at 0.827 U cm^{-2}, 5 mM, 2.5%, 5.31 mg cm^{-2}.

Table 1
Detection of the effects of some metal ions on the activity of catalase in the presence and absence of calcium ion.

Metal ions	[a]Response %	Metal ions	[a]Response %
$CaCl_2$	100	$CaCl_2$	100
$MgSO_4$	78	($CaCl_2$ + $MgSO_4$)	70
$NiCl_2$	67	($CaCl_2$ + $NiCl_2$)	40
NaCl	33	($CaCl_2$ + NaCl)	100
KCl	33	($CaCl_2$ + KCl)	90
$MnCl_2$	11	($CaCl_2$ + $MnCl_2$)	85
$CuCl_2$	–	($CaCl_2$ + $CuCl_2$)	100

[a] Average of five measurements.

3.4. Analytical characteristics of the biosensor

3.4.1. Linear range of the biosensor

The results obtained for the determination of detection limits for calcium are given in Fig. 3. When we consider the figure, it

Table 2
Determination of Ca level of some drinks by using the biosensor and reference method.

Sample	Reported (mg/l)	[a]Found (mg/l) (by the biosensor)	Recovery %	SD	[a]Found (mg/l) (by the titrimetric)	Recovery %	SD
Milk 1	120	119	99.2	±0.707	115	95.8	±2.121
Milk 2	127	126	99.2	±1.410	120.5	94.9	±1.920
Milk 3	170	167	98.2	±0.866	161.4	94.9	±2.014
Min.water 1	134.5	133	98.9	±1.000	125.8	93.5	±1.654
Min.water 2	393.2	390	99.2	±1.581	401.2	102	±2.872
Min.water 3	335	338	101	±0.707	358	106.8	±2.005
Spring water	22.2	22.4	101	±0.100	21.1	95	±0.854

[a] Average of five measurements.

can be said that the biosensor responses depended linearly on the calcium concentration between 1 and 10 mM with ($y = 0.006x + 0.046$) and $R^2 = 0.935$, the detection limit of the biosensor was determined to be 1 mM.

3.4.2. Reproducibility

The reproducibility of the biosensor was also investigated for 5 mM calcium concentration ($n = 7$). The average value (x), the standard deviation (SD) and variation coefficient (CV%) were calculated to be 5.1 mM, ±0.106 mM and 2.07%, respectively.

3.4.3. Effect of some metal ions on the activity of catalase

In order to determine the effect of different compounds on the catalase activity some experiments were carried out using 5 mM calcium and various substances such as $CaCl_2$, NaCl, KCl, $MgSO_4$, $NiCl_2$, $CuSO_4$ and $MnCl_2$ at the same concentration with calcium in the presence of 5 mM hydrogen peroxide. The increases in the biosensor response obtained with calcium was compared to other biosensor responses obtained in the presence of the other substances (Table 1).

In the other words, for the investigation of these metal ions on the catalase activity in the presence of calcium ion, some experiments were made. Table 1 also shows these results obtained from the experiments.

According to the results, although $MnCl_2$, KCl, $MgSO_4$ and $NiCl_2$ played activator role on the catalase activity in the absence of calcium ion, they showed negative effects on the catalase activity in the presence of calcium.

3.5. Application

In this section of the study the calcium content of some drinks were detected by using the biosensor. The results obtained from the biosensor were compared to results obtained using a reference procedure (Kamal, 1960) for the same samples in order to complete the validation of the new method. For this goal, some drinks such as milk, water and mineral water which contains different quantity of calcium, were used. Results obtained from the experiments were given in Table 2. From the experiments when we compare the results of two methods it can be said that calcium in the drinks can be determined sensitively by using the biosensor.

4. Conclusion

In this study, an amperometric biosensor was developed in order to investigate the effect of calcium on the activity of catalase enzyme. From the experimental studies we detected a positive effect of calcium on the enzyme activity and this effect increased in higher calcium concentrations. By using the biosensor we detected a linear concentration range for calcium in the presence of constant concentration of hydrogen peroxide. The biosensor is really original for Ca determination especially liquid samples and there is no any study like this in the literature. It does not need any expensive equipments, materials or laboratory conditions. The biosensor is portable so it can be used in everywhere for Ca analysis. For all liquid samples it is not necessary any pre-treatment for the samples except dilution (if it is necessary) in the Ca determination. The biosensor developed can be used not only food samples also clinical purpose. In dealing with a large number of samples, the biosensor is rapid, accurate, precise and with low operation cost is required. By using the biosensor because of the specificity of the enzyme we can determine calcium concentration in the presence of the other metal ions such as Cu^{2+}, Mg^{2+}, Ni^{2+}, Na^{+}, Mn^{2+} and K^{+}. Reproducibility of the biosensor is very well and from the application studies of real samples it can be said that the biosensor can be used as a sensitive alternative method for analysis of calcium.

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