Horseradish peroxidase sol–gel immobilized for chemiluminescence measurements of alkaline-phosphatase activity

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Received 30 July 2001; received in revised form 1 November 2001; accepted 7 November 2001

Abstract

2-Naphthyl phosphate is a pro-enhancer of the luminol–H2O2–horseradish peroxidase reaction. Alkaline-phosphatase hydrolyses the phosphate group and produces 2-naphthol. This compound is an enhancer of the chemiluminescence (CL) reaction. The influence of 2-naphthyl phosphate and the incubation time on the chemiluminescence reaction, have been studied. The horseradish peroxidase (HRP) was immobilized in a sol–gel matrix, obtaining a biosensor for alkaline-phosphatase. The relative standard deviations using free HRP in solution and the immobilized one were 3.2% (n = 4) and 9.3% (n = 3), respectively. The detection limits for alkaline-phosphatase using free and immobilized HRP were 128 and 106 μU/ml, respectively.

Keywords: Alkaline-phosphatase; HRP; Enhanced chemiluminescence; Biosensor; 2-Naphthyl phosphate

1. Introduction

Immobilized enzymes have advantages over soluble enzymes, in addition to the convenient handling of enzyme preparations; the two main benefits are the easy separation of the enzyme from the product and the repeated use of the enzyme [1]. The properties of immobilized enzyme are governed by the properties of both the enzyme and the carrier material. The interaction between the two results in immobilized enzyme with altered chemical, biochemical, mechanical and kinetic properties.

The immobilization of enzymes by sol–gel method is one of the fastest growing methodology for the immobilization of proteins and has the advantages of simplicity, since it not involve the need for covalent bonding between the matrix and the protein, the enhancement stability of the entrapped enzyme and the practically zero leaching of the entrapped protein. In addition, the optical transparency of the matrix, its action as a high surface area adsorbent, its chemical and photochemical inertness and the ability to obtain this novel in any desired shape and form (monoliths, thin films, powders, etc.) gives extended useful practical properties. Current problems of the sol–gel methodology are the lower reaction rates for some of the entrapped enzymes than those obtained with other techniques, and the pressure it exerts on the quaternary structure of some proteins [2].

In this work, the enzyme horseradish peroxidase is immobilized in a sol–gel matrix [3] because the optical transparency of the matrix it can be integrated with chemiluminescence (CL) spectroscopy to produce an optical biosensor to assay alkaline-phosphatase.

The determination of alkaline-phosphatase is based in the enzymatic hydrolysis of orthophosphoric monoesters to alcohols such as the hydrolysis of 2-naphthyl phosphate to 2-naphthol. This alcohol acts as an enhancer of the luminol–H2O2–horseradish peroxidase (HRP) system CL [4] and the emission intensity is an indirect measurement of alkaline-phosphatase activity.

2. Experimental

2.1. Reagents

Peroxidase from horseradish type VI-A, RZ ap. 3.0 (Sigma) was prepared in Tris–HCl buffer 0.1 M pH 8.5. Alkaline-phosphatase EC 3.1.3.1 from bovine intestine type I 4.3 U/mg (Sigma) was prepared in NaHCO3 buffer 0.1 M pH 9.5. Luminol (5-amino-2,3-dihydrophthalizine-1, 4-dione) (Sigma) 0.01 M was prepared by dissolving 0.0913 g of luminol (97%) in a small amount of NaOH
solution, adding Tris–HCl buffer 0.1 M (pH 8.5) until the final volume was 50 ml. Hydrogen peroxide 33.3% (Probus) and 2-naphthyl phosphate (Sigma) were prepared in bidistilled water to give final concentrations of 1 and 0.01 M, respectively; solutions of 2-naphthyl phosphate 10⁻⁴ M was prepared daily by dilution. TMOS was provided from Aldrich (grade 99%). The disposable poly-(methyl methacrylate) (PMMA) cuvettes were obtained from Dispolab-Kartell, 4.5 ml, four clear sides, P.N. 1961. Any PMMA cuvette with a range of transmittance, which permits CL detection at 425 nm is suitable.

2.2. Instruments

CL measurements for the batch dosage were performed in a Perkin-Elmer fluorescence spectrophotometer. The instrumental parameters were controlled by the Fluorescence Data Manager (FLDM) software (Perkin-Elmer). The luminescence spectrometer with the light source switched off was used. The apparatus was set in the phosphorescence mode with 0.00 ms delay time and 80 ms gate-time for period of light observation. The slit-width of the emission monochromator was set at 20 nm with λ_em of 425 nm and the photomultiplier voltage set manually to 700 V. An agitation system was used with free HRP in solution. However, this agitation would damage the gel, thus, no agitation was applied with immobilized HRP.

2.3. Immobilization of the enzyme

The sol–gels were prepared in a similar manner to that described by Ellerby et al. [5]. The acid-catalyzed silica sol was prepared by the sonication for 20 min, in a crystal vial, of TMOS (1.5 ml), de-ionized H₂O (320 µl) and 0.01 M HCl (30 µl).

For the gel formation 80 µl aliquot of enzyme (176 U/ml) in Tris–HCl buffer (14.08 U in gel) and 32 µl of sol were added into a disposable PMMA cuvette, and the gel was formed after 2 min.

2.4. Analytical procedure

For CL measurements, the appropriate volumes of 0.1 M Tris–HCl buffer at pH 8.5, 10⁻⁴ M 2-naphthyl phosphate and 0.043 U/ml alkaline-phosphatase were added to a disposable PMMA cuvette. After an incubation time of 10 min, appropriate volumes of 0.01 M luminol and 176 U/ml HRP were added. The CL reaction was triggered by injecting H₂O₂ with a syringe through a septum. The final volume in the cuvette is 2 ml. The CL intensity maxima are obtained from the intensity–time plots.

To obtain the gels, volumes of HRP in Tris–HCl buffer and sol were added in this order. The buffer is needed to avoid losses of enzyme activity by de-naturalization during the sol–gel process (since the pH of the sol is acid), thus, making the enzyme remain at its optimum pH [6].

3. Results and discussion

3.1. Effects of alkaline-phosphatase

2-Naphthyl phosphate acts as a pro-enhancer of the luminescence of luminol–H₂O₂–HRP system [4]. The enzyme alkaline-phosphatase catalyzes the hydrolysis of 2-naphthyl phosphate to 2-naphthol, a highly fluorescent product (λ_ex = 332 nm, λ_em = 416 nm).

The hydrolysis of 2-naphthyl phosphate catalyzed by alkaline-phosphatase was monitored observing the variation of the relative fluorescence intensity (ΔRFI) with time. The kinetic curve was used to determine the initial rate of hydrolysis. The representation of the initial rate against the substrate concentration gives a curve that was fitted to a hyperbolic shape, corresponding to Michaelis–Menten kinetic. The enzymatic parameters calculated are the Michaelis constant (K_m), 0.17 mM; and the maximum reaction rate (V_max), 15.5 ARFI/s.

The enzyme alkaline-phosphatase is active in the pH range 8–10, 9.5 being the optimum pH. In order to optimize the coupled enzymatic reaction with the luminol–H₂O₂–HRP system a pH 8.5 is chosen; this pH is the optimum for the CL system while a suitable amount of enhancer is producing (Fig. 1).

To obtain larger differences between samples with and without alkaline-phosphatase, the mixture of alkaline-phosphatase and 2-naphthyl phosphate was incubated previously at pH 8.5, then appropriate volumes of luminol and HRP were added in the same cuvette and the CL reaction triggered by injecting H₂O₂ (Fig. 2). The sample with alkaline-phosphatase shows greater intensity (curve b).

3.2. Effect of 2-naphthyl phosphate concentration

The effect of 2-naphthyl phosphate concentration on the maximum CL was studied in the range 0.25–4.00 mM

![Graph](image_url)  
Fig. 1. Relative fluorescence intensity vs. time for (●) pH 8.5 and (▲) pH 9.5. [Alkaline-phosphatase] = 32.25U/l, [2-naphthyl phosphate] = 175 µM.
The CL emission without alkaline-phosphatase is independent of the pro-enhancer concentration; with alkaline-phosphatase, the CL increases with the substrate concentration and for concentrations of 2-naphthyl phosphate greater than 2.50 mM the emission suffers a little decrease and remains constant, probably because an excess of 2-naphthol inhibits the CL emission. A 2.50 mM 2-naphthyl phosphate concentration is selected for the alkaline-phosphatase analysis because it gives the most CL intensity and permits the greater sensitivity analysis.

The enhancer of the chemiluminescent reaction, 2-naphthol, is liberated by the hydrolysis reaction. So, an incubation time is needed to obtain a suitable concentration of enhancer in the reaction medium. With 2.50 mM 2-naphthyl phosphate, the incubation time of 10 min was chosen as the optimum.

3.3. Alkaline-phosphatase assay

The calibration plot for different concentrations of alkaline-phosphatase for the selected working conditions is shown in Fig. 4. The relationship between alkaline-phosphatase concentration (mU/ml) and CL intensity maximum using blank corrected was fitted by polynomial regression of second-order. The detection limit (DL) was 128 mU/ml, the relative standard deviation (R.S.D.) of a sample with 752 mU/ml alkaline-phosphatase was 3.2% \( (n = 4) \) and the dynamic range 400–1300 mU/ml.

3.4. Alkaline-phosphatase assay using a sol–gel biosensor

The CL reaction of the luminol–H\textsubscript{2}O\textsubscript{2}–HRP system with immobilized HRP using micro encapsulation in a sol–gel...
matrix has been used to develop a biosensor for hydrogen peroxide [3] and phenolic compounds [7].

The working conditions for the HRP immobilization in a sol–gel matrix were previously studied [3]; and the working conditions for the alkaline-phosphatase assay were the same as the used before. To obtain gels, volumes of Tris–HCl buffer, enzyme in Tris–HCl buffer and sol were added in this order.

The relationship between alkaline-phosphatase concentration (μU/ml) and CL intensity maximum using blank corrected are linear in the range 215–860 μU/ml (with \( r = 0.995 \)). The obtained DL is 106 μU/ml and R.S.D. is 9.3% (\( n = 3 \)) for 537 μU/ml alkaline-phosphatase concentration (Fig. 5).

The HRP immobilization permits the linear calibration for alkaline-phosphatase, and the DL is smaller than using solution measurements, although the R.S.D. with immobilized HRP is bigger than using free HRP in solution.

The activity of the immobilized enzyme was evaluated with continuous use. It was observed that activity was rapidly lost and after five uses the enzyme showed no activity. The biosensor lifetime was investigated when it was stored in water at 4 °C. The CL signal was not stable after two months of sensor storage. However, the gel in HRP immobilized can be mass-produced, prepared and stored easily, and discarded after single use.

4. Conclusions

The enzymatic hydrolysis of 2-naphthyl phosphate (pro-enhancer) by the enzyme alkaline-phosphatase produces 2-naphthol (enhancer). This reaction is coupled to the CL reaction of the luminol–H₂O₂–HRP system. The used protocol, simultaneous incubation of two enzymatic systems, is fast and simple. One of the two enzymes involved in this reaction system has been immobilized (HRP) in a sol–gel matrix to develop a biosensor. This methodology has been applied to the biosensor obtaining good results. The two enzymatic systems have been coupled in the biosensor to assay alkaline-phosphatase giving low DL and linear calibration.

Acknowledgements

This research was supported by the DGI of MCyT (Spain), Project BQU2000-1161.

References