CHINESE JOURNAL OF CHEMISTRY 2002, 20, 1573—1578

Tetra-substituted Amino Aluminum Phthalocyanine as a New Red-region Fluorescent Substrate for Horseradish Peroxidase Based Enzyme-linked Immunosorbent Assay

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The use of tetra-substituted amino aluminum phthalocyanine (TAAlPc) as a new red-region fluorescent substrate for horseradish peroxidase (HRP)-based enzyme-linked immunosorbent assay was investigated. TAAlPc displayed an excitation maximum at 610 nm and emission maximum at 678 nm in a strong acidic medium. In the presence of HRP, trace amounts of H_2O_2 could rapidly and significantly react with TAAlPc, thus quenching the fluorescence of TAAlPc. The Michaelis-Menten parameters K_m and V_{max} were measured to be 2.82×10^{-6} mol/L^{-1} and 6.0×10^{-9} mol·L^{-1}·s^{-1}, respectively. In this paper, TAAlPc was used in an HRP-based enzyme-linked immunosorbent assay (ELISA) of α-fetoprotein (AFP) in human serum with satisfactory results. AFP could be determined in the concentration range of 0.5—200 ng/mL with a detection limit of 0.2 ng/mL, which was close to that of radioimmunoassay. The advantage of proposed method was strongly minimizing the interference resulting from background fluorescence or scattering light and had a high analytical sensitivity.

Keywords tetra-substituted amino aluminum phthalocyanine, red-region, fluorescent substrate, horseradish peroxidase, enzyme-linked immunosorbent assay

Introduction

Enzyme immunoassay based on a selective antigen-antibody binding and a label enzyme has gained increasing importance in recent years. Among the enzymes used, horseradish peroxidase (HRP) is the most widely used enzyme label because of its high specificity and sensitivity.[1-3] Fluorimetric detection possesses inherently higher sensitivity than spectrophotometric approaches. A wide variety of fluorogenic substrates are known for horseradish peroxidase (HRP),[4-10] such as tyramine, homovanillic acid, *p*-hydroxyphenylacetic acid (*p*-HPA) and *p*-hydroxyphenylpropionic acid (*p*-HPPA). However, the oxidation products of these compounds possess fluorescence excitation and emission spectra in the relatively short wavelength region of 300—420 nm. In this region, it is easily subject to the interference resulting from the background fluorescence and scattered light of the matrix. In addition, some of the substrates are expensive and not easily available. Hence, developing new fluorogenic substrates for HRP with fluorescence emission in red-region or near-infrared region, such as phthalocyanine and cyanine, which can be selectively oxidized by hydrogen peroxide catalyzed by HRP, has been a new trend in the further development of HRP-based fluorescence immunoassay.

A new promising red-region substrate, tetra-substituted amino aluminum phthalocyanine (TAAlPc, Fig. 1)

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Received November 6, 2001; revised June 12, 2002; accepted August 19, 2002.

Supported by the National Natural Science Foundation of China (No. 29775021) and the Research Fund for the Doctoral Program of Higher Education.

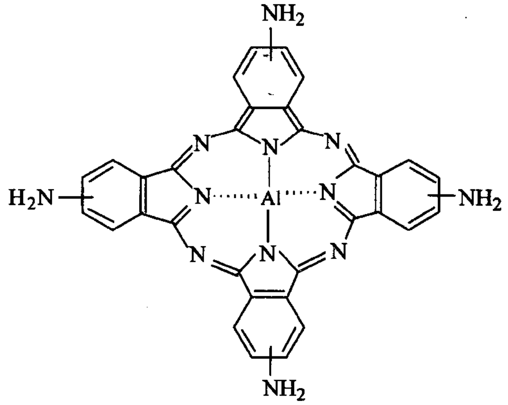

Fig. 1 Molecular structure of TAAlPc.

was synthesized.[11] TAAlPc displayed an excitation maximum at 610 nm and emission maximum at 678 nm in a strong acidic medium. In this work, TAALPc was used as a new red-region fluorescent substrate for horseradish peroxidase (HRP) based enzyme-linked immunosorbent assay (ELISA). Because of the red-region excitation and emission of this substrate, the interference from the background of biological constituents can be strongly minimized and a high sensitivity for the method is achieved. Furthermore, the fluorescent substrate is synthesized easily and stored conveniently, making this method suitable for practical applications. In the presence of HRP, trace amounts of H_2O_2 could rapidly and significantly react with TAAlPc, thus quenching the fluorescence of TAAlPc. Under optimum conditions, the calibration graph had a linear range of 0—3.94×10^{-11} mol/L for HRP with a detection limit of 5.9×10^{-13} mol/L. Based on the ELISA method, TAAlPc as a new substrate for HRP was employed for the assay of human serum α-fetoprotein (α-AFP). α-AFP was a cancer-related antigen. The level of α-AFP in the human serum was a familiar clinic test index for cancer diagnosing. Radioimmunoassay (RIA) and ELISA are the most common methods in the determination of α-AFP. In this experiment, AFP could be determined in the concentration range of 0.5—200 ng/mL with a detection limit of 0.2 ng/mL, which was close to that of radioimmunoassay.[12] The proposed method is simple, rapid and highly sensitive. To our knowledge, using a red-region or near-infrared fluorescence dye as a substrate for ELISA has not been reported previously.

Experimental

Reagents

TAAlPc was synthesized and purified according to the reported method.[11] Anal. calcd for TAAlPc ($C_{32}H_{20}N_{12}Al \cdot 2H_2O$): C 60.47, H 3.78, N 26.46; found C 60.05, H 4.05, N 25.98.

A stock solution (1.0×10^{-3} mol/L) was prepared by dissolving solid TAAlPc in redistilled dimethyl formamide. This solution was stable at room temperature for at least six months. Horseradish peroxidase (HRP, $R/Z = 3.0$, 250 U/mg) was purchased from Sigma. Its stock solution was 25 U/mL and the accurate concentration was determined at 403 nm by using a molar absorptivity of 1.02×10^5 L·mol^{-1}mL^{-1}.[13] AFP (500 ng/mL), *anti*-AFP monoclonal antibody (MAb, 5 mg/mL) and HRP-labeled *anti*-AFP polyclonal antibody (AFP-Ab) were purchased from Sino-American Biotechnology Company (Shanghai, China). Hydrogen peroxide (H_2O_2, 30 %, V/V) was obtained from Shanghai Taopu Chemical Factory (Shanghai, China), and the stock solution (3% H_2O_2, V/V) was standardized by titration with a standard solution of $KMnO_4$. 96-Well plates were obtained from Corning Glass Works (New York). All the reagents were of analytical grade. Distilled and de-ionized water was used throughout.

Apparatus

The fluorescence spectra and relative fluorescence intensity were measured with a Hitachi 650-10S fluorescence spectrophotometer with a 10-mm quartz cell. Absorption spectra were determined on a Beckman DU-7400 ultraviolet-visible spectrophotometer.

Assay procedure for HRP

1.0 mL of NaH_2PO_4-NaOH buffer solution, various amounts of HRP, 0.1 mL of TAAlPc (1.0×10^{-4} mol/L) and 0.1 mL of H_2O_2 (0.01 mol/L) were placed in a 10 mL of volumetric tube. The mixture was quickly diluted to 5.0 mL with water and allowed to stand for 3 min at room temperature. Then 0.5 mL of HCl (3.0 mol/L) was added and diluted to the mark. Finally, the relative fluorescence intensity of the solution was measured at 678 nm with an excitation wavelength of 610 nm.

Immunoassay

The 96-well plate was coated with *anti*-AFP monoclonal antibody (MAb). The MAb solution was first diluted to 5 μg/mL with sodium carbonate buffer (0.1 mol/L, pH 9.6) and 200 μL of the diluted MAb solution was then placed into wells of the plate and incubated at 4 ℃ overnight. After being washed twice with Tris-HCl buffer (0.05 mol/L, pH 7.8) containing 0.05% (*V/V*) Tween 20 (buffer 1), the wells were further washed with Tris-HCl buffer (0.05 mol/L, pH 7.8) without Tween-20 (buffer 2). Each well was blocked with 100 μL of phosphate (0.01 mol/L) buffered saline (PBS, pH 7.4) containing 1% (*W/V*) BSA, 0.05% (*W/V*) NaN_3 (buffer 3) at room temperature for 1 h. The coated plate was washed as previously and stored at − 10 ℃.

The standard solutions of AFP for calibration were prepared by diluting human AFP standard solution with buffer 3. Human serum samples were diluted 5-fold with buffer 3 prior to analysis.

The immunoassay procedure for AFP using TAAlPc as the substrate was as follows. To a coated well was added 100 μL of standard AFP solution or 100 μL of diluted human serum sample solution, and the solution was incubated for 1 h at 37 ℃. The well then was washed twice with buffer 1 and once with buffer 2. 50 μL of HRP-Ab solution (diluted by 1∶500 with buffer 3) was incubated in the well for 1 h at 37 ℃. The wells were then washed twice with buffer 1 and once with buffer 2. Then 50 μL of NaH_2PO_4-NaOH buffer solution, 50 μL of TAAlPc (5.0×10^{-6} mol/L) and 50 μL of H_2O_2 (5.0×10^{-4} mol/L) were added. The mixture was allowed to stand for 3 min at room temperature and then 50 μL of HCl (1.0 mol/L) was added. The final solution was transferred to the fluorescence spectrophotometer to measure the fluorescence intensity at 678 nm with an excitation wavelength of 610 nm.

Results and discussion

Spectral characteristics

Fig. 2 is the absorption spectra of TAAlPc in the presence of H_2O_2 in hydrochloric acid medium. Similar to other phthalocyanine, TAAlPc has two absorption bands, a soret band (in the short wavelength region) and a Q band (in the long wavelength region). When different concentrations of H_2O_2 were mixed with TAAlPc, no significant wavelength shift occurred. However, the absorption peak of both Soret band and Q band obviously decreased with increasing the amounts of H_2O_2. The excitation and emission spectra of HRP-TAAlPc-H_2O_2 and HRP-TAAlPc systems in acidic media were shown in Fig. 3. It can be seen that the fluorescence emission peak of TAAlPc was located at 678 nm. When excited at the Soret band, the secondary scattered light could cause interference; on the other hand, it was easily subject to the interference resulting from the impurity fluorescence of the background matrix. Therefore, 610 nm was chosen as the excitation wavelength. As can be seen from Fig. 3, the fluorescence spectra of the HRP-TAAlPc-H_2O_2 system are similar in shape to those of HRP-TAAlPc system, but the fluorescence intensity of the former system is dramatically decreased. In order to explore the reason of the fluorescence quenching of TAAlPc, other three phthalocyanines, tetra-substituted sulphonated aluminum phthalocyanine (AlS4Pc), tetra-substituted carboxyl aluminum phthalocyanine (AlC4Pc) and tetra-substituted phenylthio aluminum phthalocyanine (AlP_4Pc) were also checked. The results showed that only the fluorescence of TAAlPc could be greatly quenched by H_2O_2 in the presence of HRP. It can be concluded that the fluorescence quenching of TAAlPc might be due to the oxidation of the amino groups of TAAlPc by H_2O_2 under the catalysis of HRP.

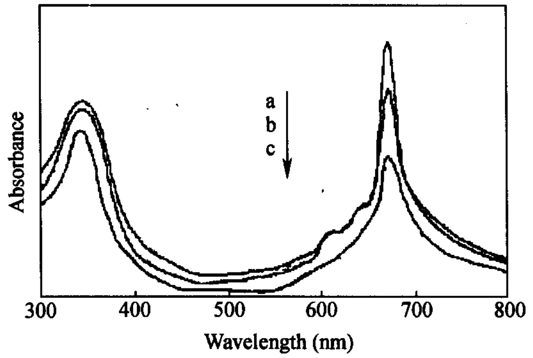

Fig. 2 Absorption spectra of TAAlPc. TAAlPc, 1.0×10^{-6} mol/L; HRP, 3.94×10^{-9} mol/L. Concentration of H_2O_2: a—0, b—1.0×10^{-7} mol/L, c—5.0×10^{-7} mol/L.

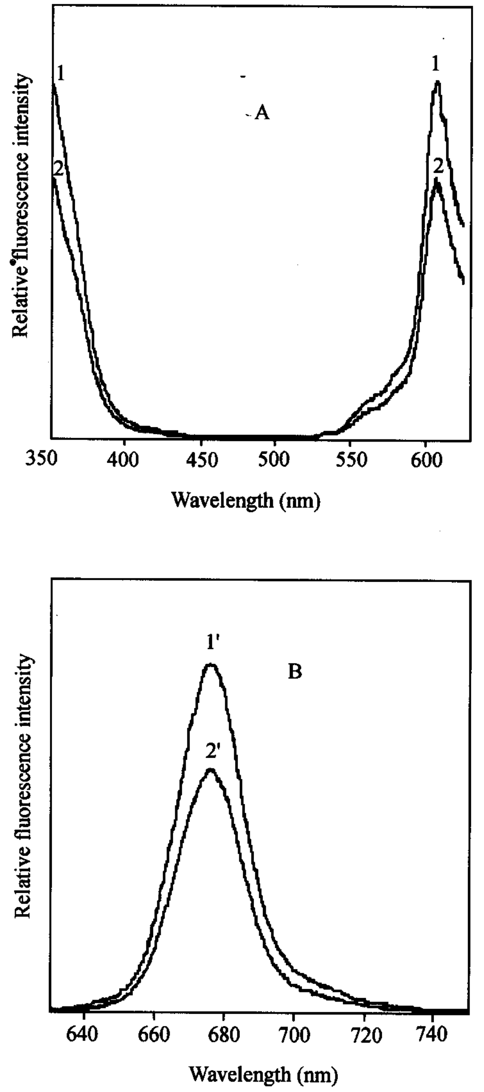

Fig. 3 Fluorescence excitation spectra (A) and emission spectra (B) of HRP-TAAlPc (1, 1′) and HRP-TAAlPc-H_2O_2(2, 2′) systems. HRP, 3.94×10^{-9} mol/L; TAAlPc, 1.0×10^{-6} mol/L; H_2O_2, 1.0×10^{-7} mol/L.

Selection of optimal conditions for HRP-*catalyzed reaction*

A fluorimetric method based on an HRP-catalyzed reaction usually has two acidity requirements, one is suitable for the HRP-catalyzed reaction and the other is suitable for getting the greatest fluorescence change of the reaction system. Our experiments showed that the maximum fluorescence quenching for the HRP-TAAlPc-H_2O_2 system occurred at pH 6.0 for the HRP-catalyzed oxidation reaction of TAAlPc. The impact of various buffers on the HRP-TAAlPc-H_2O_2 reaction system was also examined, and the results showed that the activity of HRP in NaH_2PO_4-NaOH medium was much higher than that in hexamethylenetetramine-HCl and Tris-HCl buffers. Therefore, a pH of 6.0 obtained by adding 1.0 mL of NaH_2PO_4-NaOH buffer solution (0.05 mol/L) was recommended for HRP determination. As to the acidity required to obtain the greatest fluorescence change of the reaction system, experiments indicated that the fluorescence quenching was maximum and constant when the concentration of HCl in the system was varied from 0.06 to 0.24 mol/L. Thus, a HCl concentration of 0.15 mol/L was employed in subsequent work.

The influence of reaction time on the fluorescence quenching of TAAlPc in the HRP-catalyzed oxidation reaction was also investigated. The result showed that the fluorescence intensity was strong enough for measurement after the reaction had proceeded for 3 min. Here, the fixed-time method with 3 min was chosen for the determination of HRP.

The effect of temperature on the fluorescence intensity of the system is slight from 10 to 40 ℃. Therefore, the reaction can be performed at room temperature.

The optimal amounts of TAAlPc and H_2O_2 were also studied. The fluorescence quenching of TAAlPc was maximum and constant when the final concentration of H_2O_2 was in the range of 5×10^{-5}—2×10^{-4} mol/L. Therefore, an H_2O_2 concentration of 1×10^{-4} mol/L was adopted. Results showed that the lower the concentration of TAAlPc, the higher the sensitivity of the method would be, but at the expense of the linear range. Thus, the final concentration of TAAlPc was set at 1.0×10^{-6} mol/L, taking both sensitivity and linear range into account.

Calibration graph for HRP

The calibration curve for the determination of HRP was constructed under the optimal conditions. A good linear relationship was observed between the fluorescence intensity and the HRP concentration in the range of 0.0—3.94×10^{-11} mol/L. The limit of detection was calculated by the equation LOD = $K\sigma/S$, where K is a numerical factor chosen according to the confidence level desired, σ is the standard deviation of the blank measurements ($n = 12$) and S is the sensitivity of the calibration graph. Here a valve of 3 for K was used. The detection limit for HRP was calculated to be 5.9×10^{-13} mol/L.

The correlation coefficient was 0.996 ($n = 7$). The relative standard deviation was 0.77% ($n = 8$) for the determination of 9.85×10^{-12} mol/L HRP.

Effects of interfering substances

The interference effects of some foreign substances on the determination of HRP (1.00×10^{-11} mol/L) were examined and the results are summarized in Table 1. It can be seen that only Co^{2+}, Mn^{2+}, Ni^{2+}, Sn^{2+} and Hg^{2+} ions could cause serious interference. However, when the system is used for an HRP-based enzyme-linked immunosorbent assay, the human serum samples should be highly diluted, hence the metal ions listed above would not cause interference because of the trace level of concentration in the samples. The results also showed that albumin, globulin and hemoglobin in serum samples do not affect the assay.

Measurement of constants for the enzymatic reaction

The catalytic activity of HRP in the reaction of TAAlPc with H_2O_2 was studied by the initial-rate method with the steady-state assumption where the concentration of H_2O_2 was saturated in the test system. The Michaelis-Menten constant K_m and the transformation constant K_{cat} were obtained from the equation of $V_{max} = K_{cat} \cdot [E_0]$, where E_0 is the initial concentration of the enzyme, K_{cat} represents the catalytic activity. The greater the K_{cat}, the greater the enzymatic activity would be. The Michaelis-Menten parameters K_m and V_{max} were measured to be 2.82×10^{-6} mol·L^{-1} and 6.0×10^{-9} mol·L^{-1}·s^{-1}. The K_{cat} was 61 s^{-1} relative to the enzyme concentration of 9.85×10^{-11} mol·L^{-1}.

Calibration graph for AFP

The calibration curve for the determination of AFP was constructed under the optimal conditions. The result is shown in Fig. 4. A good linear relationship was observed between the fluorescence intensity and the AFP concentration over the concentration range of 0.5—200 ng/mL. The detection limit for AFP was 0.2 ng/mL given by the calculation with the equation LOD = $K\sigma/S$. The correlation coefficient was 0.996 ($n = 9$).

The stability of the method with time was also examined. The reagents used in the method were stored at 4 ℃ for two months. Then a calibration curve for the determi-

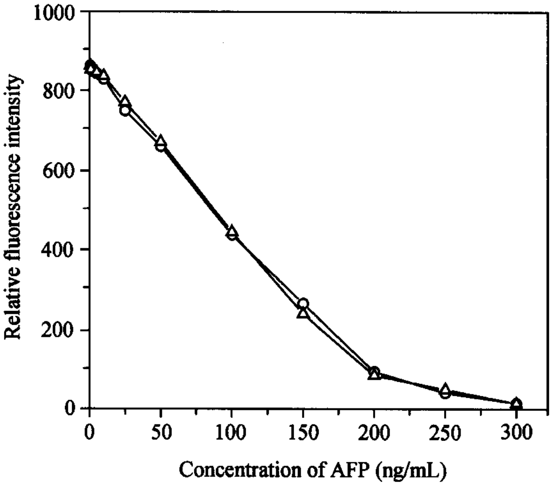

Fig. 4 Calibration curves for the determination of AFP (○) after 0 day and (△) after two months.

Table 1 Tolerance of interfering substances in the determination of 1.00×10^{-11} mol/L HRP

Coexisting substances	Coexisting conc. ($\times 10^{-4}$ mol/L)	Relative error (%)	Coexisting substances	Coexisting conc. ($\times 10^{-4}$ mol/L)	Relative error (%)
Ca^{2+} (chloride)	25	+4.9	Ni^{2+} (chloride)	0.01	+2.9
Al^{3+} (chloride)	1	+4.2	Co^{2+} (sulphate)	0.01	+3.6
Mg^{2+} (chloride)	1	+5.4	Cu^{2+} (sulphate)	0.02	+3.4
K^{+} (chloride)	200	+2.7	Sn^{2+} (chloride)	0.01	+4.9
NH^{4+} (chloride)	100	+3.7	n^{2+} (chloride)	0.01	+5.0
Zn^{2+} (chloride)	5	+4.0	EDTA	0.5	−5.1
Cd^{2+} (chloride)	10	+4.6	HSA	0.05	−5.3
Pb^{2+} (chloride)	0.1	+8.6	IgG	0.05	−4.3
Fe^{2+} (sulphate)	2	+3.2	Hemoglobin	0.01	+7.4
Fe^{3+} (sulphate)	0.02	+6.3			

Table 2 Determination of AFP in the sera of colon carcinoma patients

Patient sera	AFP levels[a] (ng/mL)	RSD ($n = 12$, %)	Added (ng)	Found (ng)	RSD ($n = 12$, %)
1	535	2.5	10	9.2	6.2
2	394	6.8	10	11.5	5.8
3	502	7.3	10	9.3	7.1
4	479	4.2	10	11.1	6.6
5	457	5.5	10	8.8	7.5

[a] Mean of twelve determinations.

nation of AFP was constructed again under the optimal-conditions as described above (Fig. 4). The result shows that the stability of this method with time is good.

Immunoassay of AFP *in human blood sera*

The AFP levels in sera of colon carcinoma patients were determined by the proposed assay. The results are summarized in Table 2. The relative standard deviation within a batch was below 10% ($n = 12$). The possibility of using the proposed method for the analysis of real samples was further confirmed by determining the recovery of known amounts of AFP added to the samples. The results in Table 2 show that the recovery and reproducibility of the proposed method are satisfactory.

It can be concluded from the above results that the synthesized tetra-substituted amino aluminum phthalocyanine is a promising substrate for an HRP-based enzyme-linked immunosorbent assay. The method offers the advantage of being sensitive because of using a red-region fluorescent reagent, and being possible to select the desired chemical structure of substrate since it is based on the use of a synthesized metal-complex. In this sense, further exploitation of its applications in the field of biochemical analysis is promising.

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(E0111067 LU, Y. J.; DONG, L. J.)