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Evaluation of a new substrate for measurement of serum PON arylesterase activity

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ABSTRACT

It was found that the hydrolysis of 9-(4-chlorophenyloxycarbonyl)-10-methylacridinium triflate (CPOCMA) could be catalyzed by recombinant human PON1. Based on this property, the CPOCMA was evaluated as a substrate for serum PON activity assay. The apparent K_m value of a serum sample for the substrate was determined as 85 nmol/L, close to the K_m value (83 nmol/L) of rHuPON1. The interferences by other esterase such as acetylcholinerase and lipases were investigated. The NaCl and $CaCl_2$ as PON activity enhancers were able to improve the specific signal, respectively. The rHuPON1 in presence of $CaCl_2$ showed at least 7.8 times selectivity over acetylcholinerase and lipases. By comparing with the UV methods based on phenyl acetate and diazinon, respectively, the proposed chemiluminescent method was validated with 30 serum samples. The method based on CPOCMA allows reliable, cost-saving, and specific determination in a buffer of physiological pH.

1. Introduction

Paraoxonase (PON), a high–density lipoprotein-associated hydrolyase, is able to hydrolyze lipid peroxides in LDL and homocysteine thiolactone, which are regarded to be risk factors of atherosclerosis in vitro studies [1]. In addition to atherosclerosis, PON has exhibited multifaceted functions in predicting and preventing other diseases, such as organophosphate detoxification, endometrial cancer [2,3], potential bladder cancer [4], hepatitis C virus infection [5], gestational diabetes mellitus [6,7], depression [8], and cardiac syndrome X [9], etc. Serum PON levels decrease in haemodialysis patients and renal transplantation patients has been observed recently [10,11]. In an excellent review paper, Goswami and Furlong described the important roles that PON plays in inhibiting various diseases [1,12]. The artificial substrates used for PON activity assay are mainly classified into three categories as lactones, organophosphates, and arylesters. Of the substrates, lactones are generally used to evaluate the PON lactonase for atherosclerosis assessment, and the organophosphates and arylesters are widely used in evaluation of nearly all PON-related diseases. The organophosphorous esters such as paraoxon, diazinon and chlorpyrifos show good specificity toward PON. The determination is based on the PON-catalyzed hydrolysis of the substrate, and the hydrolysis product is monitored by an increase in UV absorption. However, the substrates show very low turnover rate under the catalysis of PON in a buffer of physiological pH. Even in a buffer pH 8.0 the assay based on paraoxon is not sensitive due to inefficiency of the catalysis and low UV absorbance of the hydrolysis product 4-nitrophenol, so some measurements were performed in a buffer pH 10.5 [13–15]. Such basic pH buffer possibly leads to alteration of PON structure and thus its activity. The 7-diethylphospho-6,8-difuor-4-methylumbelliferyl (DEPFMU) was developed as a substrate for PON activity assay [16,17]. The activity was measured according to an increase in fluorescence emitted by the hydrolysis product of DEPFMU. Due to high sensitivity of fluorescence, the low turn-over rate of DEPFMU could be accurately detected. Therefore, the PON assay based on DEPFMU could be easily performed in a buffer pH 8.0, and the sensitivity was improved compared with the method based on paraoxon. However, the determination was also performed in a basic pH buffer. Generally most of organophosphorous compounds are highly toxic.

Phenyl acetates are a class of carboxyl esters, showing quick turnover rate in a buffer pH 8.0 under the catalysis of PON. The phenyl acetate and the 4-nitrophenyl acetate have been the most widely used artificial substrates for PON arylesterase assay. This class of substrates has advantages of quick turnover rate, low cost, easy availability, and low toxicity. However, phenyl acetates are not specific toward PON, and their hydrolysis can be catalyzed by a number of other serum esterases. It was reported that the assay based on the phenyl acetate had a high background signal of 35–50% [17].

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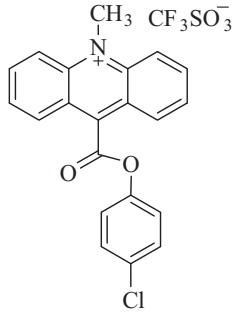


Fig. 1. The molecular structure of CPOCMA.

Acridinium esters are well-known highly chemiluminescent compounds [18–20]. The hydrolysis products of acridinium esters are non-chemiluminescent. Therefore, the hydrolysis of acridinium esters can be monitored by a decrease in chemiluminescence. The super sensitive detection of acridinium esters allows easy determination of the hydrolysis, and thus the reaction based on an acridinium ester for PON activity assay can be performed in a buffer of physiological pH. Compared with the currently used substrate, acridinium esters are characteristic of big molecular size, bulky substituents linked the carbonyl group, and good hydrophilicity. Such properties are important for specificity of a substrate. As we know, acridinium esters have not been included in the family of PON substrates. In this paper, the 9-(4-chlorophenyloxylcarboxyl)-10-methylacridinium triflate ester (CPOCMA) which is a member of acridinium esters, would be investigated as a PON substrate to achieve reliable PON arylesterase activity assay under physiological pH condition.

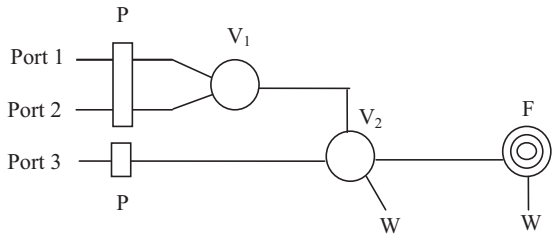
2. Materials and methods

2.1. Apparatus

A Ruimai IFFM-E flow-injection chemiluminescence machine (Xi'an, China) was used for chemiluminescence tests. ^{1}H NMR spectra were measured on a Bruker 500 spectrometer with tetramethylsilane as the internal standard.

2.2. Materials

The chemical structure of CPOCMA is presented in Fig. 1. It was prepared according to the literature [20]. ^{1}H NMR (DMSO-d_6, δ, ppm): 4.96 (s, 3H), 7.73 (d, 2H, J = 9 Hz), 7.85 (d, 2H, J = 9 Hz), 8.18 (dd, 2H, J = 9, 7 Hz), 8.56 (dd, 2H, J = 9, 7 Hz), 8.66 (d, 2H, J = 9 Hz), 8.95 (d, 2H, J = 9 Hz). The phenyl acetate was self-prepared. Pure rHuPON1 was purchased from Ximei Chemical Ltd (Shanghai, China), which was prepared by ProSpec (Protein-Specialists), East Brunswick. The molecular weight as 45 kDa of rHuPON1 was assumed from literature [21] to calculate the molar concentration. Lipidase and acetylcholinesterase were purchased from Lanji Technology Co. Ltd (Shanghai, China) and Gelin Biotechnology Co. Ltd (Fuzhou, China), respectively. The diazinon was purchased from Zhongyijiaxin Technology Co. Ltd (Tianjin, China). Tris, $CaCl_2$, NaCl, H_2O_2, CTAB, HNO_3 and NaOH were of analytical grade. Distilled-deionized water was used through out. Human blood sera were freshly collected from healthy people in routine physical examination in Hospital of Chongqing University, and was stored in a refrigerator at 4 °C and used up within 5 days.



Scheme 1. The chemiluminescent test procedure for the flow-injection mode: P, peristaltic pump; V_1, three-way valve; V_2, six-way valve; W, waste solution; F, flow cell for detection; port 1, test solution; port 2, triggering reagent 1 ($HNO_3 + H_2O_2$); port 3, triggering reagent 2 (NaOH + CTAB).

2.3. Methods

2.3.1. Chemiluminescent tests

The stationary mode of chemiluminescence test was used the in Section 3.3, and flow-injection mode was used for the tests in the other sections. The procedure for a flow-injection mode was shown in Scheme 1. The test solution and triggering reagent 1 *via* port 1 and port 2, respectively, are driven by peristaltic pump into a blender room, and then the mixed solution passes through a six-way valve. By changing the valve passage position, triggering reagent 2 *via* port 3 passing through the six-way valve is introduced into the same passage of the above mixture. By self-diffusion, the triggering reagent 2 meets the above mixed solution, and the chemiluminescence is triggered. The chemiluminescence intensity is recorded automatically. The procedure for stationary mode: to a test beaker into the detecting room of the machine, a test solution (20 μL), triggering reagent 1 (20 μL), and triggering reagent 2 (1 mL) were introduced subsequently. The chemiluminescent kinetics was recorded and the chemiluminescence intensity was calculated by the integral of peak area.

2.3.2. Methods for PON activity assay

A requisite amount of CPOCMA was dissolved in acetonitrile to prepare the stock solution (1.1×10^{-3} mol/L). The CPOCMA stock solution stored in a refrigerator was diluted with a Tris–HCl buffer (0.1 mol/L, pH 7.5) before incubation with serum at 25 °C. Unless otherwise stated, the final substrate concentration was 8.27×10^{-8} mol/L and the final serum concentration was 1250-fold diluted. Afterwards, the chemiluminescence was tested. A series of blank solutions in absence of serum were made and their chemiluminescence intensities were recorded. 1 U of arylesterase activity is defined as 1 μmol of CPOCMA hydrolysis per minute.

The procedure for PON activity assay based on phenyl acetate was performed according to an established method as follows. The assay mixture included 500-fold diluted serum, 1.0 mmol/L $CaCl_2$ in 0.1 mol/L Tris–HCl, pH 8.0, and phenyl acetate of 6.84×10^{-3} mol/L. The test solution was measured spectrophotometrically at 270 nm before incubation for 10 min. One unit of activity is equal to 1 mmol of phenyl acetate hydrolyzed per minute [15].

The method for PON activity assay with diazinon was performed by modifying the established procedure [13–15] as follows. The basal assay mixture contained 1.0 mM diazinon of 1.29×10^{-3} mol/L and 1.0 mM $CaCl_2$ in 0.1 mol/L Tris–HCl buffer pH 8.0. One unit of PON activity is defined as 1 mmol of diazinon hydrolyzed per minute.

3. Results and discussion

3.1. The pH optimization

The serum-mediated hydrolyses of CPOCMA in different pH buffers of 6.0, 7.0, 7.5, 7.8, 8.0, 8.1, 8.2, 8.3 and 8.5 were determined,

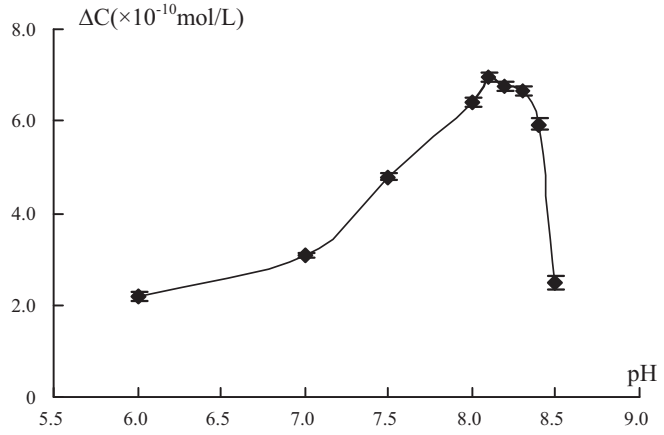


Fig. 2. The serum-mediated hydrolyses of CPOCMA under different pH buffers. The investigated pH ranged from 6.0 to 8.5. The CPOCMA of 1.1×10^{-9} mol/L and the serum of 1250-fold dilution in pH buffer were subjected to incubation for 10 min at 25 °C before chemiluminescence test.

respectively. The results are shown in Fig. 2. It is clear that the serum PON showed the best catalytic ability for the hydrolysis of CPOCMA in buffer pH 8.0. This result was consistent with the optimal pH for PON assay based on phenyl acetate [15,16,22–24]. However, the self-hydrolysis of CPOCMA was clearly observed at basic conditions (pH ≥ 8.0). Moreover, the serum PON activity measured under basic condition might not be in accordance with the real case in a biological system. Therefore, the buffer pH 7.5 in place of buffer pH 8.0 used for the measurement would resolve the problem. The sensitive chemiluminescent method based on CPOCMA enables to detect serum-mediated CPOCMA hydrolysis in pH 7.5 buffer. Therefore pH 7.5 would be selected for the following investigations.

3.2. The reaction kinetics

To ensure the PON activity assay is reasonable, the incubation must be over before the reaction equilibrium arrives. The reaction kinetics is very important for setting a reasonable incubation period, and during which the reaction must conform to one-order reaction kinetics.

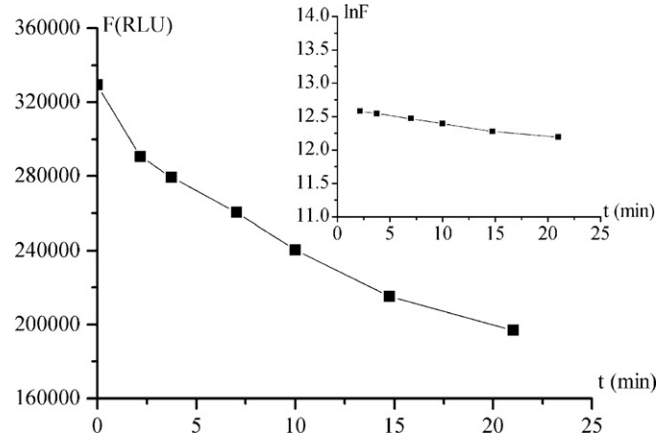


Fig. 3. The kinetic curve for the rHuPON-catalyzed hydrolysis of CPOCMA in a buffer pH 7.5 at 25 °C. The CPOCMA concentration was 2.90×10^{-8} mol/L; the rHuPON concentration was 6.34×10^{-10} mol/L.

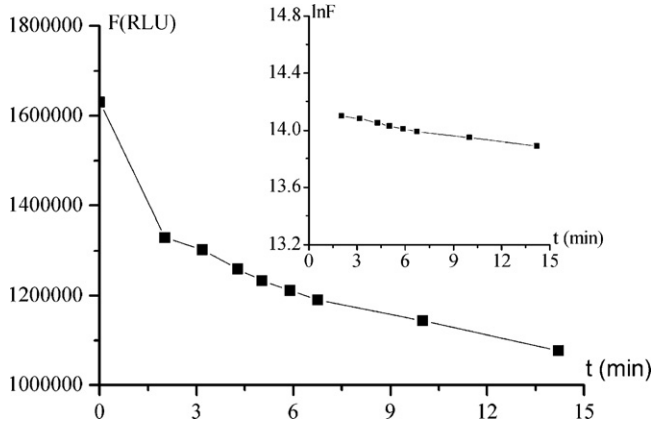


Fig. 4. The kinetic curve for the serum-mediated CPOCMA hydrolysis in a buffer pH 7.5 at 25 °C. The CPOCMA concentration was 8.27×10^{-8} mol/L; the serum concentration was 1250-fold diluted.

3.2.1. The rHuPON-catalyzed hydrolysis of CPOCMA

The reaction kinetics for the rHuPON-catalyzed hydrolysis of CPOCMA was shown in Fig. 3. The reaction kinetics obeyed one-order kinetic equation as follows.

$$c = c_0 \exp(-kt) \quad \text{(I)}$$

In Eq. (I), c is the CPOCMA concentration during incubation; c_0 is the initial concentration of the CPOCMA; k is a constant of reaction velocity; t is the reaction period of the CPOCMA hydrolysis. Due to that the chemiluminescence intensity is proportional to CPOCMA concentration, Eq. (I) can be converted into Eq. (II):

$$F = F_0 \exp(-kt) \quad \text{(II)}$$

In Eq. (II), F is the chemiluminescence intensity of the test solution; F_0 is the chemiluminescence intensity of the CPOCMA before incubation with serum or rHuPON. Eq. (III) can be deduced from Eq. (II) through a mathematical conversion:

$$\mathrm{Ln}\, F = -kt + \mathrm{Ln}\, F_0 \quad \text{(III)}$$

Therefore, the kinetic curve can also be seen in the inset of Fig. 3 which was obtained by plotting $\mathrm{Ln}\, F$ against t. A linear equation of $\mathrm{Ln}\, F = -0.0102 \times t + 12.616$ ($n = 6$, $r = 0.9960$) was observed. From Eq. (III) we know that the line slop should be the constant of the reaction velocity, and it was calculated as 0.0102 min^{-1}.

3.2.2. The reaction kinetics of serum-mediated hydrolysis of CPOCMA

The reaction kinetics of serum-mediated CPOCMA hydrolysis was shown in Fig. 4. As expected, the chemiluminescent signal decayed with time going, and the decay also conformed to one-order kinetic equation. The $\mathrm{Ln}\, F$ was plotted against t to give the inset of Fig. 4. The linearity was observed from the inset of Fig. 4, and a linear equation of $\mathrm{Ln}\, F = -0.00620 \times t + 14.123$ ($n = 8$, $r = 0.9972$) was obtained. The line slope indicates the constant of the reaction velocity being 0.00620 min^{-1}.

The kinetic curve shows that the reaction fitted first-order kinetics model. It implies the chemiluminescence decrease was mainly due to enzyme-catalyzed CPOCMA hydrolysis rather than other quenchers in serum, since if the chemiluminescence decrease had been caused by serum quenchers, the decrease should have stopped within a very short period.

3.3. Determination of K_m

K_m value is a parameter for evaluating the affinity between the substrate and the enzyme. Its value is dependant on substrate

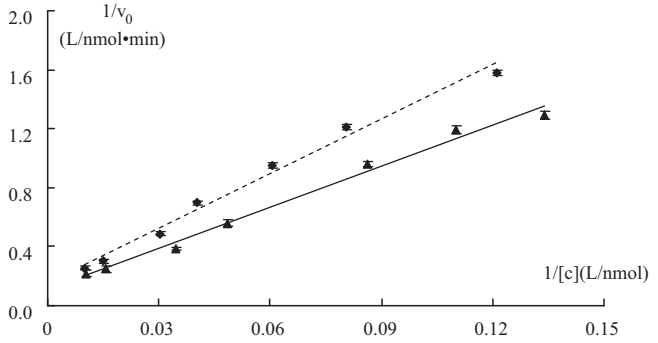


Fig. 5. Lineweaver–Burk plots. (1) The solid line is for the rHuPON-catalyzed COPCMA hydrolysis in a buffer pH 7.5 at 25 °C. The rHuPON concentration was fixed at 6.34×10^{-10} mol/L. The COPCMA concentrations were 7.45×10^{-9}, 9.10×10^{-9}, 1.16×10^{-8}, 2.07×10^{-8}, 2.92×10^{-8}, 6.45×10^{-8}, and 9.55×10^{-8} mol/L, respectively. (2) The dashed line is for the serum-mediated COPCMA hydrolysis in a buffer pH 7.5 at 25 °C. The serum concentration was 1250-fold diluted. The COPCMA concentrations were 8.29×10^{-9}, 1.24×10^{-8}, 1.65×10^{-8}, 2.48×10^{-8}, 3.31×10^{-8}, 6.62×10^{-8}, 9.93×10^{-8} mol/L, respectively.

properties and reaction conditions such as temperature and pH buffer, but it is independent of enzyme concentration. To obtain K_m value, the reciprocal of the initial velocity of the hydrolysis $1/v_0$ as a function of $1/c$ was plotted. The reaction velocity can be calculated according to Eq. (IV) as follows, which is derived from Eq. (I):

$$v = -\frac{dc}{dt} = c_0 k \exp(-kt). \quad \text{(IV)}$$

When t is 0, the reaction velocity v is the initial reaction velocity, which can be calculated as $v_0 = c_0 \cdot k$.

The data presented in the curve of solid line in Fig. 5 for determination of rHuPON1 K_m obeyed classical Lineweaver–Burk plot, and A linear equation of $1/v_0 = 9.28 \times (1/[c]) + 0.112$ ($n = 7$, $r = 0.9949$) was obtained. The reciprocal of the apparent K_m of rHuPON1 was obtained from the intercept on the abscissa of the solid line. The apparent K_m of rHuPON1 using CPOCMA as a substrate was calculated as 83 nmol/L.

The data for determination of apparent serum K_m obeyed classical Lineweaver–Burk plot, and they were presented as the dashed line in Fig. 5. A linear equation of $1/v_0 = 12.4 \times (1/[c]) + 0.146$ ($n = 7$, $r = 0.9941$) was obtained. The reciprocal value of the intercept (K_m value) on the abscissa was calculated as 85 nmol/L. The value was close to that of the rHuPON1. It means that the PON in the serum were mainly responsible for the CPOCMA serum-mediated hydrolysis. The difference of the K_m values between of the rHuPON and of the serum resulted from PON1 polymorphism, the physiological location of the enzyme (PON is associated with HDL), the naturally occurring calcium and sodium in the serum, diet, and the other unknown physiological conditions [17,25]. Moreover, the apparent K_m value of the serum was possibly affected by the other serum esterase present in the serum. In fact, the variation of apparent K_m values was observed in different serum samples from different people.

The K_m of rHuPON1 (83 nmol/L) for CPOCMA is much lower than the K_m values for the other substrates, such as 91 μmol/L of PON1 $L_{55}R_{192}$ and 200 μmol/L of PON1 $L_{55}Q_{192}$ for DEPFMU, respectively [17], 420 μmol/L for phenyl acetate, 300 μmol/L for diazoxon, 360 μmol/L for chlorpyrifos oxon, and 540 μmol/L for paraoxon [26]. The low K_m value is possibly due to the special molecular structure and the reaction conditions (such as low reaction temperature, and buffer of low pH).

3.4. Improvement of serum-mediated CPOCMA hydrolysis by PON enhancers

It has been reported that sodium chloride and calcium chloride are PON activity enhancers. They have been used to improve the specific signal and sensitivity [22–30] in serum PON activity assay. Herein, the two PON activity enhancers were used to boost the serum-mediated CPOCMA hydrolysis. If the serum-mediated CPOCMA hydrolysis is mainly attributed to serum PON, it would be expected that the $CaCl_2$ and NaCl would improve the serum-mediated CPOCMA hydrolysis significantly. Otherwise, the $CaCl_2$ and NaCl would not improve the serum-mediated CPOCMA hydrolysis. The effects of NaCl and $CaCl_2$ were investigated in the following section, respectively.

Fig. 6A depicts the serum-mediated CPOCMA hydrolysis under the influence of NaCl and $CaCl_2$, respectively. As expected, NaCl and $CaCl_2$ each were able to enhance the serum-mediated CPOCMA hydrolysis significantly. As the concentrations of PON enhancers increasing, the serum-mediated CPOCMA hydrolyses within 10 min were boosted, respectively, but the changing rate attenuated.

The boosting effect by NaCl and $CaCl_2$ was further characterized by comparing the constants of reaction velocity before and after addition of the NaCl (40 mmol/L) and $CaCl_2$ (1.2 mmol/L), respectively. The Ln F was plotted against t to obtain Fig. 6B. In Fig. 6B the solid line is for the serum-mediated CPOCMA hydrolysis without any PON enhancer; the dotted line is for the NaCl-boosted serum-mediated CPOCMA hydrolysis; the dashed line is for the $CaCl_2$-boosted serum-mediated CPOCMA hydrolysis. The constants of reaction velocity k after addition of NaCl and $CaCl_2$ were calculated as 0.010 min^{-1} and 0.011 min^{-1}, respectively. By comparing the k value of the reaction without addition of a PON enhancer, we know that the NaCl and the $CaCl_2$ boosted the constant of reaction velocity as 1.6 times and 1.7 times, respectively.

In all, as expected, serum-mediated hydrolyses of CPOCMA could be boosted significantly by adding PON agonists such as NaCl and $CaCl_2$, respectively. The result is consistent with our previous speculation that serum PON was mainly responsible for the serum-controlled CPOCMA hydrolysis. Otherwise, the serum-mediated hydrolysis could not be affected by PON activity enhancers.

3.5. Interference

In addition to PON, other serum esterases might catalyze the CPOCMA hydrolysis [31], and they would cause non-specific activity in PON activity assay. Representative esterases would be investigated, since it is impossible to investigate all hydrolyses. It was estimated that the carboxyl esterases mainly contributed to the non-specific serum activity, while other hydrolyses such as protease or amidase would display poor catalytic ability toward a carboxyl ester as CPOCMA. Therefore interferences from lipase and acetylcholinesterase were evaluated, respectively.

Fig. 7 shows the kinetic curves for the acetylcholinesterase-catalyzed CPOCMA hydrolysis and the lipase-catalyzed CPOCMA hydrolysis, respectively. In Fig. 7, from the dashed line we know that the acetylcholinesterase of 0.630 nmol/L did not show any catalytic activity toward the CPOCMA hydrolysis within 20 min; from the solid line it is clear that the lipase accelerated the CPOCMA hydrolysis evidently. The constant of the reaction velocity (k) was calculated as 0.00220 min^{-1} (on the condition that the lipase concentration was 0.63 nmol/L) according to the linear equation of $\text{Ln}\, F = -0.00220 \times t\,(\text{min}) + 14.319$ ($n = 6$, $r = 0.8860$). Through comparison of the velocity constants of the rHuPON-catalyzed CPOCMA hydrolysis and that of the lipase-catalyzed one, we know that the rHuPON displayed 4.6 times selectivity over the lipase. Combining the boosting effect of $CaCl_2$ in Section 3.4. PON in presence of $CaCl_2$ (1.2 mmol) demonstrated 7.8 times selectivity over lipase.

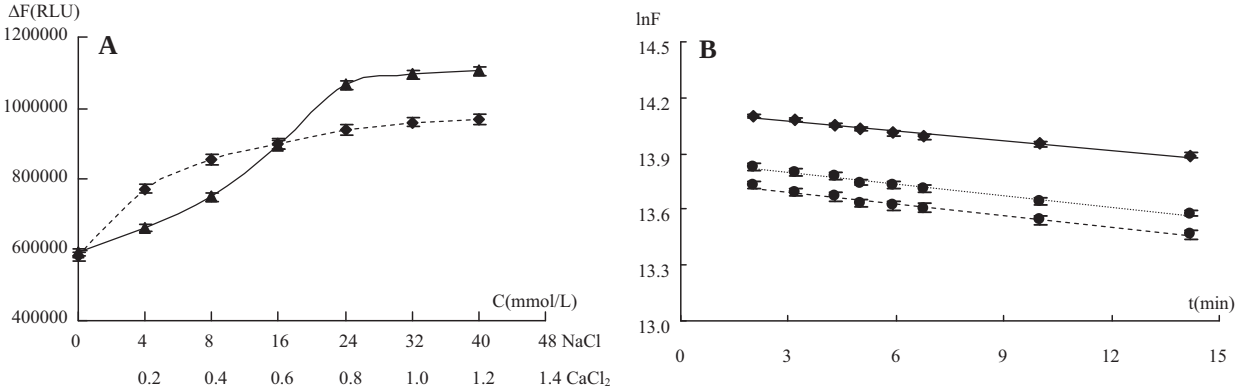


Fig. 6. (A) The improved serum-mediated CPOCMA hydrolysis by NaCl (the dashed line) and $CaCl_2$ (the solid line), respectively. The NaCl concentration ranged from 4 mmol/L to 40 mmol/L, and the $CaCl_2$ concentration ranged from 0.2 mmol/L to 1.2 mmol/L. The CPOCMA concentration of 8.27×10^{-8} mol/L was fixed and the serum was 1250-fold diluted. The incubation was set for 10 min at 25 °C. (B) The reaction kinetics. (1) The solid line is for the serum-mediated CPOCMA hydrolysis without any PON enhancer; (2) the dotted line is for the improved serum-mediated CPOCMA hydrolysis by NaCl; (3) the dashed line is for the improved serum-mediated CPOCMA hydrolysis by $CaCl_2$.

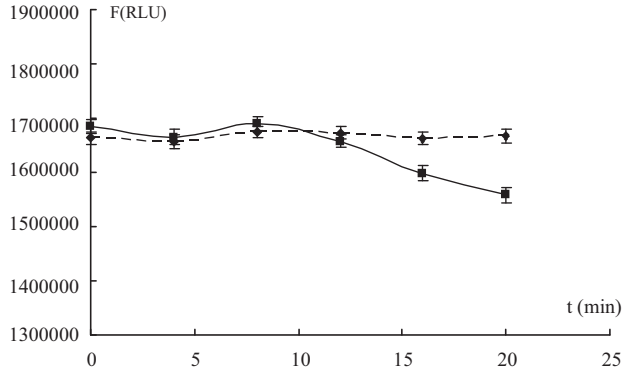


Fig. 7. The reaction kinetic curves for the acetylcholinesterase-catalyzed (the dashed line) and the lipase-catalyzed (the solid line) CPOCMA hydrolyses, respectively. The CPOCMA concentration was fixed at 8.27×10^{-8} mol/L, and both the concentrations of acetylcholinesterase and lipase were 0.630 nmol/L (25 °C, Tris–HCl buffer pH 7.5). The assay solution was tested at intervals.

Fig. 8 depicts the CPOCMA hydrolysis catalyzed by the esterases of different concentrations within 10 min. It shows that the acetylcholinesterase (the dashed line) was not able to boost CPOCMA hydrolysis even when its concentration was as high as 3.15 nmol/L, which is as 5 times high as the rHuPON1 concentration used in Section 3.2.1. This result is consistent with that shown in Fig. 7. Moreover, addition of $CaCl_2$ or NaCl could not change the acetylcholinesterase activity toward the substrate (the data are not presented in this paper). It means that the acetylcholinesterase in serum would not cause interference in PON activity assay based on the CPOCMA.

From the solid line in Fig. 8, we know as the lipase concentration increasing, the CPOCMA hydrolysis increased. Further studies show that the $CaCl_2$ or NaCl could not improve the lipase-controlled CPOCMA hydrolysis, or could not improve the lipase activity (the data are not shown in this paper).

From the above discussion, we know that the serum lipase was responsible for the serum non-specific activity. Addition of NaCl or $CaCl_2$ did not improve the activities of lipase and acetylcholinesterase toward the substrate hydrolysis, and therefore PON activity enhancers can increase specificity and sensitivity of the serum PON-mediated signals. The PON boosted by $CaCl_2$ demonstrated at least 7.8 times selectivity over lipase and acetylcholinesterase. It means the serum PON was mainly responsible for the observed serum-mediated CPOCMA hydrolysis.

3.6. Linearity

According to the optimized conditions, the linearity was determined by plotting constant of the reaction velocity k against serum concentration. The data are presented in Fig. 9. A linear equation of $k = 0.0062 \times c + 0.0013$ (μL/mL serum) ($n = 6$, $r = 0.9959$) was obtained in the serum concentration range of 250–5000-fold dilution. The linearity was also observed by plotting the Ln $F(t = 10\ \text{min})$ against the serum concentration.

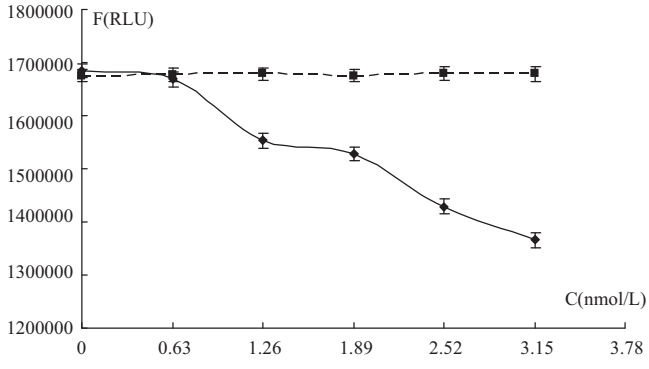


Fig. 8. The CPOCMA hydrolysis catalyzed by the esterases of different concentrations. The dashed line and the solid line are for the catalyses of acetylcholinesterase and of lipase, respectively. The CPOCMA concentration was 8.27×10^{-8} mol/L, and the reaction period was fixed for 10 min (25 °C, pH 7.5 Tris–HCl buffer).

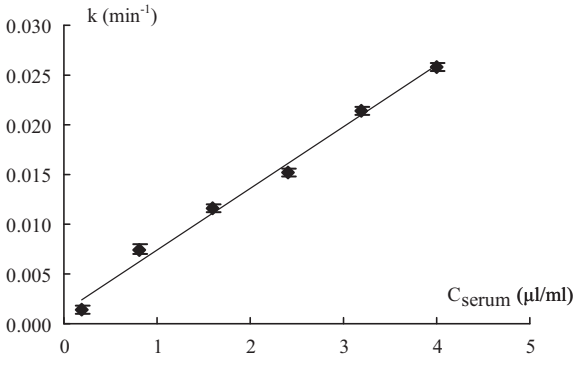


Fig. 9. The calibration plot for the serum-mediated hydrolysis of CPOCMA. The serum was 5000-, 1250-, 625-, 400-, 312-, and 250-fold diluted, respectively. The CPOCMA concentration of 8.27×10^{-8} mol/L and the $CaCl_2$ concentration of 2.0 mmol/L were used. The chemiluminescence intensity was measured at interval of 4 min.

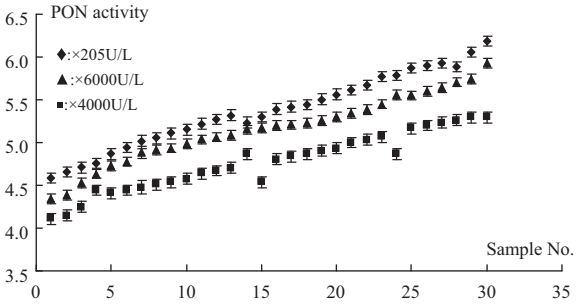


Fig. 10. Serum PON activities of 30 samples based on (1) phenyl acetate (♦); (2) CPOCMA (▲); (3) diazinon (■).

3.7. Validation

The proposed chemiluminescent method based on the CPOCMA for serum PON activity assay was validated with 30 random serum samples by comparing with two UV methods based on phenyl acetate and diazinon, respectively. The procedures for the PON activity assay are presented in Section 2.3, but the procedure for the chemiluminescent method in Section 2.3 was slight modified. The PON enhancer ($CaCl_2$ of 2.0 mmol/L) was included in the incubation solution, and the incubation was fixed for 10 min. Fig. 10 presents the PON activities of 30 samples determined using three methods. The samples are numbered sequentially according to the PON activity determined with the CPOCMA. The symbols "♦ ▲, and ■" are for the results based on phenyl acetate, CPOCMA, and diazinon, respectively. As we know, for each sample the absolute units of the PON activities based on different substrates are incomparable. However, the three curves in Fig. 10 are of a similar trend more or less. It seems that for the most of the serum samples the result measured by the chemiluminescent method was consistent with the results obtained by the UV methods based on phenyl acetate or diazinon. The several exceptive cases might result from PON polymorphism. It proposed that the chemiluminescent method should be reliable.

3.8. Variation coefficient

Reproducibility tests showed that the within-day RSD and the day-to-day RSD of the serum PON activity were less than 1.7% ($n = 9$) and less than 5.6% ($n = 5$), respectively, for 3 serum samples.

4. Conclusion

There were several evidences showing that the CPOCMA was a reliable and improved substrate of PON. The PON boosted by $CaCl_2$ showed more than 7.8-times selectivity over acetylcholinesterase and lipase toward the CPOCMA hydrolysis. The PON enhancers such as NaCl and $CaCl_2$ were able to boost the rHuPON-mediated or serum-mediated hydrolysis of CPOCMA, respectively, but they were not able to boost the lipase-mediated or acetylcholinesterase-mediated CPOCMA hydrolysis. Therefore, addition of PON enhancers can improve specificity and sensitivity.

In this proposed chemiluminescent method, due to high chemiluminescent efficiency of the CPOCMA, the used substrate concentration was at least 1.0×10^5 times lower than that of phenyl acetate used in the classic UV method. The reduction of substrate amount in the assay could result in cost-saving and waste-saving. And this method allows the assay performance in a buffer of physiological pH without sacrificing sensitivity.

In the near future, the specificity and sensitivity of other acridinium esters as PON substrates will be investigated.

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