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Article

Facile Chemiluminescence Assay for Acyl-CoA Oxidase Activity: Fundamentals and Illustrative Examples

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A acil-CoA oxidase (ACO) é a enzima-chave que catalisa a etapa determinante da velocidade da beta-oxidação peroxissomal de ácidos graxos. A ACO catalisa a oxidação de acil-CoA com oxigênio molecular, produzindo trans-2,3-desidroacil-CoA e H_2O_2. Considerando a participação de H_2O_2 em uma reação quimiluminescente melhorada com luminol, nós desenvolvemos um procedimento de duas etapas novo para quantizar a atividade de ACO usando um detector de quimiluminescência para registrar a emissão de luz. São apresentadas várias recomendações de padronização das condições de reação para a medida quantitativa das reações catalizadas por ACO. O método proposto é simples e confiável, e foi aplicado com sucesso ao teste com fígado de rato. Os resultados mostraram que as atividades de ACO eram maiores no fígado de rato com diabetes tipo 2, mas não apresentaram alteração no fígado de rato resistente à insulina.

Acyl-CoA oxidase (ACO) is the key enzyme that catalyzes the rate-determining step in the peroxisomal beta-oxidation of fatty acids. ACO catalyzes the oxidation of acyl-CoA with molecular oxygen to produce trans-2,3-dehydroacyl-CoA and H_2O_2. Given the participation of H_2O_2 in an enhanced chemiluminescent reaction with luminol, we have developed a novel "two-step" procedure for the quantitation of ACO activity by recording light emission using a chemiluminescence detector. A number of recommendations on standardizing the reaction conditions for the quantitative measurement of ACO-catalyzed reactions are offered. The proposed method is simple and reliable and has been successfully applied in the rat liver assay. The results indicate that ACO activities increased in the liver of type-2 diabetic rats but showed no significant change in the liver of insulin-resistant rat.

Keywords: acyl-CoA oxidase, chemiluminescence, luminol, peroxisomal oxidation

Introduction

Fatty acid degradation primarily occurs through the β-oxidation cycle. In mammals, β-oxidation occurs in the mitochondria and peroxisomes. When the fatty acid chains exceed the length that can be handled by the mitochondria (more than C-22), fatty acid β-oxidation occurs in the peroxisomes. Peroxisomal β-oxidation consists of four steps: (1) dehydrogenation, (2) hydration, (3) redehydrogenation, and (4) thiolytic cleavage. These reactions are catalyzed by different enzymes (Figure 1). In peroxisomes, the first reaction of the β-oxidation spiral is catalyzed by acyl-CoA oxidase (ACO), which, in contrast to the mitochondrial acyl-CoA dehydrogenases, directly reduces oxygen to hydrogen peroxide (H_2O_2). ACO is the initial and rate-limiting enzyme of the peroxisomal β-oxidation system. Recently, increasing evidence suggests that reduced ACO activity can cause fatty acid β-oxidation deficiency, which leads to fatty acid accumulation.[1-3] Thus, the measurement of ACO activity has received increasing attention.

Several spectrophotometric and fluorometric methods have been developed to measure ACO activity. Small *et al.*[4] coupled the formation of H_2O_2 to the oxidation of a dye that can be assayed by monitoring the changes in A502 using a recording spectrophotometer. This spectrophotometric method was used to measure the ACO activities in the liver of brown trout (*Salmo trutta*)[5] and mammals.[6] Kvannes *et al.*[7] developed a method for assaying peroxisomal fatty acyl-CoA oxidase in subcellular

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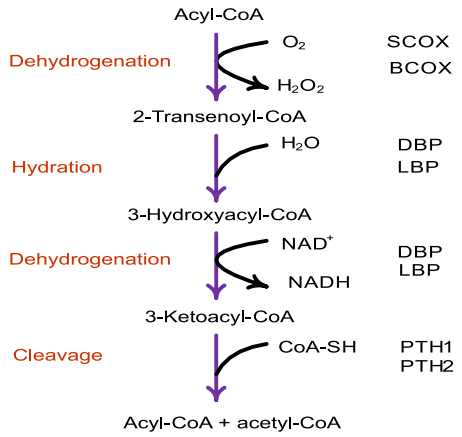

Figure 1. Process and enzymology of fatty acid β-oxidation in human peroxisomes. SCOX: straight chain acyl-CoA oxidase; BCOX: branched chain acyl-CoA oxidase; DBP: D-bifunctional protein; LBP: L-bifunctional protein; PTH: peroxisomal thiolase.

fractions. The method was based on the peroxidase-linked oxidation of 4-hydroxyphenylacetic acid to a fluorescent compound. By utilizing indolepropionyl-CoA as a chromogenic substrate, Gopalan *et al.*[8] measured the ACO activity either directly, by monitoring the formation of the reaction product indoleacryloyl-CoA (λ_{max} = 367 nm), or indirectly, by measuring the formation of H_2O_2 via the oxidative-coupled assay system, which involves 4-aminoantipyrine, phenol, and horseradish peroxidase (HRP). However, despite their previous development, these assays still suffer from a number of drawbacks, including poor sensitivity in measuring small volumes of samples and the need for derivatization.

Chemiluminescence (CL) has attracted considerable attention as a versatile and highly sensitive detection tool that can be used in diverse fields, including biochemistry, bioimaging, and analytical technology. The major advantages of modern CL methods over routine biochemical assays are their high sensitivity, rapidity, and relatively low costs.[9-11] Of the different CL reagents available, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) appears to be the most popular. Luminol is oxidized by strong oxidants in the presence of a catalyst to produce CL emissions. This property has led to the use of luminol in various analytical methods. The oxidative CL of luminol has wide application in the determination of various oxidants as well as of the analytes that produce these oxidants. The use of luminol in these methods has allowed enzymes such as uricase, xanthine oxidase, and L-amino acid oxidase to be assayed.[12-14]

In the first stage of fatty acid β-oxidation in the peroxisomes, ACO catalyzes the oxidation of acyl-CoA with molecular oxygen to produce trans-2,3-dehydroacyl-CoA and H_2O_2 (c.f. E1). Therefore, the activity of ACO can be determined by measuring the amount of H_2O_2 that forms during the oxidase-catalyzed reaction. Despite the numerous advantages of luminol-based CL assays over routine procedures for oxidase monitoring, these methods also have inherent limitations, including the complexity of the CL mechanism and the high nonspecific sensitivity of these methods toward even trace impurities. In the subsequent sections, we disclose the methodology of a "two-step" design for monitoring the CL emissions in oxidation processes. This design overcomes the aforementioned problems.

Experimental

Reagents and equipment

HRP, palmitoyl-CoA, flavin adenine dinucleotide (FAD), coenzyme A (CoA), and luminol were purchased from Sigma (St. Louis, MO, USA). 4-Iodophenol (PIP, Alfa Aesar), nicotinamide adenine dinucleotide (NAD, Biomol), sodium azide, 30% H_2O_2, sucrose, $Na_2EDTA{\cdot}2H_2O$, NaOH, K_2HPO_4, KH_2PO_4, sodium dodecyl sulfate, potassium sodium tartrate, and Folin & Ciocalteu's phenol reagent were purchased either from Beijing Chemical Reagent Company (Beijing, China) or from Tianjin Chemical Reagent Company (Tianjin, China). All reagents were either analytical-grade or guaranteed reagents and used as received without further purification. The PIP solution was stored in brown glass stoppered flask at 4 °C. All buffers were prepared with deionized water and filtered through a 0.22 μm membrane prior to use.

CL measurements were performed using a flow-injection analyzer detection system (IFFM-E, Xi'an Remex Electronic Science-tech, Xi'an, China). The reaction vial was placed in front of the detection window of the photomultiplier tube. The CL signals were processed by a personal computer. A Vortex-Genie Model K 550G mixer (Scientific Industries, New York, USA) was used to mix the solutions. A Mettler Toledo FE20 pH meter was used for all aqueous pH measurements. Temperature was controlled within 40 ± 0.5 °C using a conventional control equipment and a circulating pump.

Sample preparation

For ACO activity measurements, rat tissue homogenates were prepared by placing tissues in an ice-cold saline solution to remove blood. All subsequent procedures were performed at 0 °C to 4 °C. Minced tissues were suspended in a 3.0 mmol L^{-1} Tris-HCl buffer containing 0.25 mmol L^{-1} sucrose, and 1.0 mmol L^{-1} $EDTANa_3$ (pH 7.0). The tissues were then homogenized with an electric homogenizer. The

resulting homogenate was centrifuged at 30,000 × g for 10 min at 4 °C. The supernatant was then collected for the subsequent ACO activity analysis.

Method principles and analytical procedure

The method principle and procedure are shown in Figure 2. In this assay, the difference in the CL intensities in steps 1 and 2 was used as a measure of the H_2O_2 produced during the ACO-catalyzed reaction and, consequently, of the ACO activity. Given that luminol can react with a number of coexisting substances in the liver tissue, the following "two-step" procedure was used in the ACO activity assay:

Step 1: A microglass vial containing 20 µL of a composite phosphate buffer (pH 8.5) was placed in front of the photomultiplier tube (PMT) in a light-tight detector container containing palmitoyl-CoA, FAD, 200 µmol L^{-1} NAD, 170 µmol L^{-1} CoA, 4 mmol L^{-1} NaN_3, and 5 mmol L^{-1} $EDTANa_3$. Considering the wide distribution of peroxidases among living organisms, peroxidase inhibitors (NaN_3, Na_2EDTA) were added into the reaction system to protect H_2O_2. Subsequently, 10 µL of the homogenate supernatant was added into the vial to initiate the enzymatic reaction at 40 ± 0.5 °C. Afterward, 70 µL of a CL reagent (containing 40 µmol L^{-1} luminol, 50 µg mL^{-1} HRP, and 20 µmol L^{-1} PIP) was added into the mixed solution. The resulting CL signal was immediately recorded.

Step 2: In another microglass vial containing 20 µL of a composite phosphate buffer (pH 8.5, same content and concentration as that used in step 1), another 10 µL of the homogenate supernatant was added into the vial to initiate another enzymatic reaction at the same temperature. The reaction mixture was then incubated for 10 min. Afterward, 70 µL of the CL reagent (same content as that used in step 1) was added into the mixed solution, and the resulting CL signal was immediately recorded. The difference in the CL intensities in steps 1 and 2 was due to the H_2O_2 produced during the ACO-catalyzed reaction. The CL signal in step 1 was used as the background value. One unit (U) of ACO activity was defined as the amount of enzyme that catalyzes the production of 1 µmol of H_2O_2 *per* minute under a given condition.

The luminol-H_2O_2 reaction system works optimally at about pH 11.[15] However, the high pH condition required by the luminol-H_2O_2 reaction to produce light efficiently is incompatible with enzymatic reactions. Consequently, an enhancer must be added to the luminol-H_2O_2 CL reaction mixture under mild pH conditions. Given that HRP is a poor catalyst for luminol oxidation, compounds known as primary enhancers, including PIP, are added to the substrate mixture to increase the CL intensity.[8] PIP has been used as a CL enhancer in the direct detection of liposome-encapsulated HRP using luminol CL.[8,16]

$$\text{Acyl-CoA} + O_2 \xrightarrow{ACO} \text{trans-2,3-dehydroacyl-CoA} + H_2O_2 \quad (1)$$

$$H_2O_2 + \text{luminol} \xrightarrow{HRP,PIP} \text{3-aminophthalate} + h\nu \quad (2)$$

$$\text{ACO activity (U)} = \frac{Concentration\ of\ H_2O_2\ (\mu mol\ L^{-1}) \times 10^{-3}}{Reaction\ Time\ (min)} \quad (3)$$

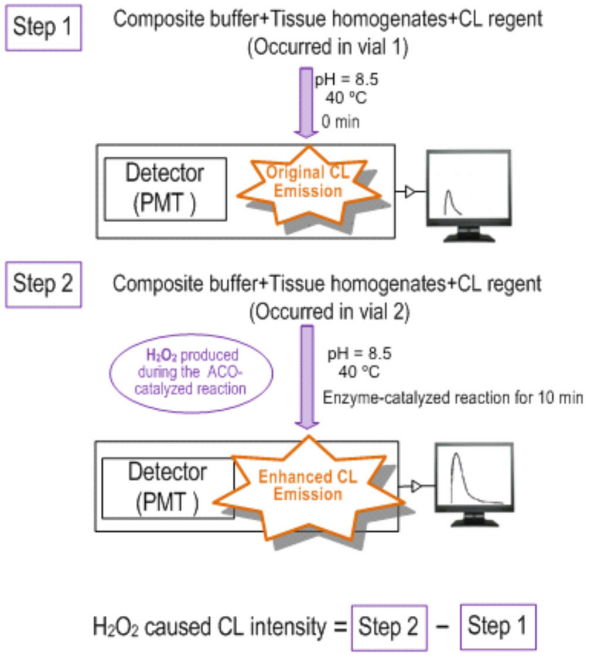

Figure 2. Analytical procedure for the acyl-CoA oxidase activity assay.

Results and Discussion

Effects of the palmitoyl-CoA concentration on the CL intensity

Palmitoyl-CoA is often used as a substrate in ACO activity assays. Rat liver peroxisomes contain three acyl-CoA oxidases: palmitoyl-CoA oxidase, pristanoyl-CoA oxidase, and trihydroxycoprostanoyl-CoA oxidase. Short-chain mono (hexanoyl-) and dicarboxylyl (glutaryl-)-CoAs and prostaglandin E2-CoA are exclusively oxidized by palmitoyl-CoA oxidase. Meanwhile, long-chain mono (palmitoyl-) and dicarboxylyl (hexadecanedioyl-)-CoAs are oxidized by palmitoyl-CoA oxidase. The very long-chain lignoceroyl-CoA is also oxidized by palmitoyl-CoA oxidase and pristanoyl-CoA oxidase. The substrate dependences of palmitoyl-CoA oxidase and pristanoyl-CoA oxidase are highly similar when assayed with the same (common) substrate.[17]

In this study, the palmitoyl-CoA concentrations in the solutions significantly affected the CL emission intensities. As shown in Figure 3, the CL intensity initially increased and then decreased as the palmitoyl-CoA concentration was increased. The inhibitory effect appears to be due to the excess substrate. The maximum CL intensity was obtained when the palmitoyl-CoA concentration was 30 μmol L^{-1}. Therefore, this concentration was chosen for subsequent studies.

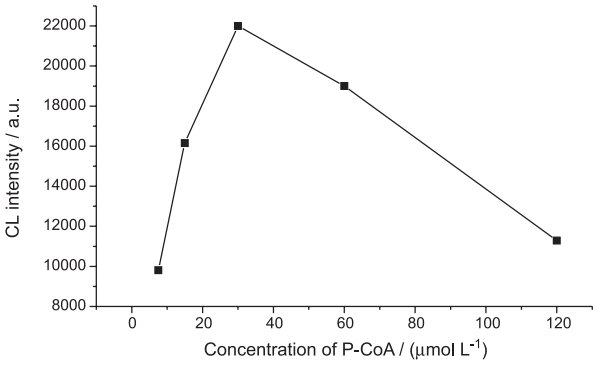

Figure 3. Effect of palmitoyl-CoA (P-CoA) concentration on chemiluminescence (CL) intensity.

Effects of the FAD concentration on the CL intensity

FAD is a cofactor of ACO and has an enhancing effect on the luminol-H_2O_2 CL system.[5] As shown in Figure 4, the FAD signals showed slight fluctuations from 10 to 90 μmol L^{-1}. These results indicate that the "two-step" design can significantly reduce the effects of FAD. Given that the 10 μmol L^{-1} FAD concentration used in this study is similar to that found in the human body, this concentration was used in our measurements.

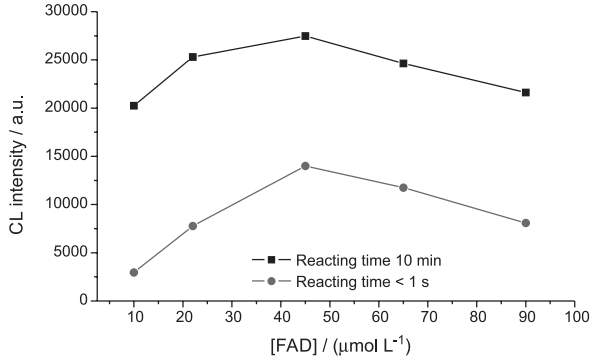

Figure 4. Effect of flavin adenine dinucleotide (FAD) concentration on chemiluminescence (CL) intensity.

Effect of the reaction time on the CL intensity

Figure 5 shows the effect of the reaction time (from 3.0 to 13.0 min) on the CL intensity. The strongest CL signal was obtained when the ACO-catalyzed reaction time was 10 min. This phenomenon is similar to that described in a previous study.[8] Therefore, the reaction time of 10 min was used in subsequent experiments.

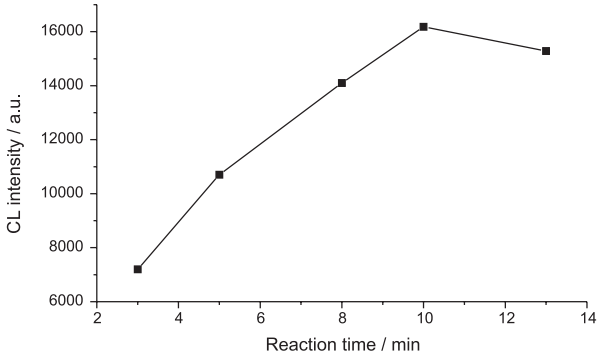

Figure 5. Effect of acyl-CoA oxidase (ACO)-catalyzed reaction time on chemiluminescence (CL) intensity.

Optimum pH

The effects of the potassium phosphate buffer pH on CL emission intensities were also determined. When the potassium phosphate buffer pH was increased from 7.0 to 10.0, the CL intensity increased and reached its peak at 8.5. Further increasing the pH reduced the CL emissions. Hence, pH 8.5 was used in the assay procedure.

Effect of the sample protein concentration on CL intensity

Protein is a coexisting substance in homogenate samples. Bovine serum albumin (BSA) was used as a standard to determine the sample protein concentrations using the method of Lowry.[18] The ACO activity in the liver tissue was expressed as the amount of milliunits *per* mg of protein (mU mg^{-1}). The results show that the CL intensity decreased as the BSA concentrations were increased from 25 to 800 μg mL^{-1} (Figure 6). The regression equation used is $I_{CL} = -14.499C + 46590$ (C is the concentration of BSA, in μg mL^{-1}). This equation indicates that 200 μg mL^{-1} BSA will slightly reduce approximately 6.8% of the CL intensity. However, the protein concentrations in the reaction system are generally less than 100 μg mL^{-1}. Therefore, the protein concentration in our sample is less than 100 μg mL^{-1}, and the effect of the protein on the assay results can be considered negligible.

Validation of the CL method

Under the optimized conditions, the linearity of the detection was assessed by triplicate analyses of solutions containing 3.3 to 60 μmol L^{-1} H_2O_2. The coefficients

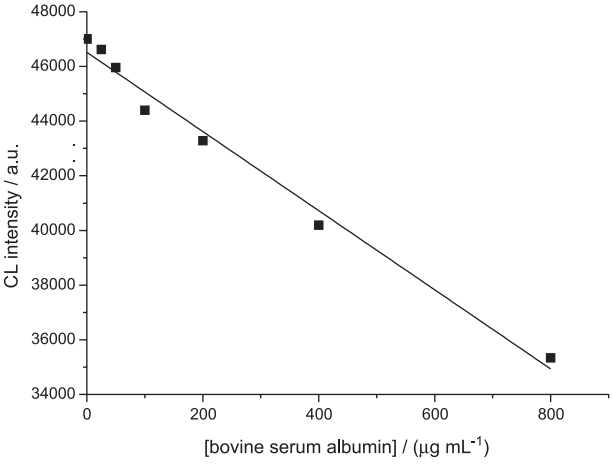

Figure 6. Effect of protein concentration on chemiluminescence (CL) intensity.

of determination ranged from 0.9980 to 0.9989. This result indicates an excellent linearity of response that is compatible with a fully quantitative method. The coefficient of variation (day-to-day) for the method was 7.6%.

Application to rat liver samples

The CL intensities of the sample solutions were detected under the same optimum conditions. The results obtained by the proposed method are shown in Table 1. Compared with that of the control groups (65.57 ± 9.16 mU mg^{-1}), the ACO activity in the liver of the diabetes groups increased (78.33 ± 12.18 mU mg^{-1}, $p < 0.05$), whereas that in the insulin-resistant groups showed no change (60.75 ± 10.15 mU mg^{-1}, $p > 0.05$) (Figure 7).

Table 1. ACO activities in rat liver

Group	n	ACO activity / (mU mg^{-1})
Con	10	65.57 ± 9.16
IR	8	60.75 ± 10.15
DM	8	78.33 ± 12.18[a]

[a]$p < 0.05$ compared with the Con group. Con: normal rats; IR: insulin resistance rats; DM: diabetic rats.

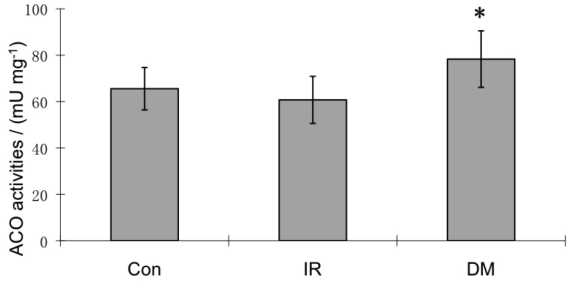

Figure 7. ACO activities in the liver of normal rats (Con), insulin resistance rats (IR), and diabetic rats (DM). *$p < 0.05$ compared with the control group.

Discussion

The "two-step" design achieved minimal interference from the coexisting substrate. The experimental conditions, including FAD and BSA concentrations, for CL detection were investigated in detail to achieve maximum assay sensitivity. The optimum conditions of the substances in the system are as follows: 30 μmol L^{-1} palmitoyl-CoA, 10 μmol L^{-1} FAD, 200 μmol L^{-1} NAD, 170 μmol L^{-1} CoA, 4 mmol L^{-1} NaN_3, and 5 mmol L^{-1} $EDTANa_3$ in 20 μL of the composite phosphate buffer (pH 8.5); and 40 μmol L^{-1} luminol, 50 μg mL^{-1} HRP, and 20 μmol L^{-1} PIP in 70 μL of the CL reagent. The ACO activity in the rat liver can be assayed by utilizing the facile CL method. The proposed method is sufficiently sensitive for the study of peroxisomal β-oxidation activities in the liver and other organs.

Most of the CL reactions of luminol with H_2O_2 occur in basic medium. Many substances, particularly a number of metal ions even at trace levels, affect the CL systems of luminol in a basic solution. In the proposed procedure, a pH 8.5 buffer is used. In such a mild pH condition, metal ions in the tissue homogenate would have negligible effects on the luminol-H_2O_2 system and thus lead to high stability and measurement reproducibility. Furthermore, H_2O_2 is fairly stable in such a mild pH solution because the decomposition of H_2O_2 begins with the hydroperoxide ion (HO_2^-), which is more readily formed in basic solutions based on the acid-base equilibrium ($pKa = 11.7$) of H_2O_2.[19]

ACO activity is most commonly assayed using a procedure in which H_2O_2 formation is coupled to the oxidation of a dye that can be spectrophotometrically measured.[4] However, the main drawback of this spectrophotometric method is its relatively low sensitivity. Therefore, we developed a highly sensitive method for ACO activity assay. The results obtained by the proposed method indicate that the ACO activities increased in the liver of type-2 diabetes rats but showed no significant changes in the liver of insulin-resistant rats. As expected, the ACO activities determined by this assay are considerably higher than those previously obtained via spectrophotometric detection.[20]

Conclusions

This study is the first to report to the use of a CL method for ACO activity determination. Under optimized conditions, the developed "two-step" procedure minimizes the interference from coexisting substrates. Moreover, the proposed method exhibits higher sensitivity and requires smaller amounts of samples compared with the previously reported spectrophotometric and fluorimetric assays.

Acknowledgement

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References

1. Kovacs, P.; Stumvoll, M.; *Best Pract. Res. Clin. Endocrinol. Metab.* **2005**, *19*, 625.
2. Kashyap, S.; Belfort, R.; Gastaldelli, A.; *Diabetes* **2003,** *52*, 2461.
3. Katarzyna, P.; Marlene, B.; *Eukaryotic Cell* **2006**, *5*, 1847.
4. Small, G. M.; Burdett, K.; Connock, M. J.; *Biochem. J.* **1985**, *227*, 205.
5. Rocha, M. J.; Rocha, E.; Resende, A. D.; Lobo-da-Cunha, A.; *Biochem.* **2003**, *4*, 2.
6. Poosch, M. S.; Yamazaki, R. K.; *Biochim. Biophys. Acta* **1986**, *884*, 585.
7. Kvannes, J.; Flatmark, T.; *J. Biochem. Biophys. Meth.* **1991**, *23*, 135.
8. Gopalan, K. V.; Srivastava, D. K.; *Anal. Biochem.* **1997**, *250*, 44.
9. Das, S.; Powe, A.; Baker, G.; Valle, B.; El-Zahab, B.; Sintim, H. O.; Lowry, M.; Fakayode, S.; McCarroll, M.; Patonay, G.; Li, M.; Strongin, R.; Geng, M.; Warner, I.; *Anal. Chem.* **2012**, *84*, 597.
10. Weizmann, Y.; Cheglakov, Z.; Willner, I.; *J. Am. Chem. Soc.* **2008**, *130*, 17224.
11. Lin, J. M.; Arakawa, H.; Yamada, M.; *Anal. Chim. Acta* **2003**, *169*, 485.
12. Seitz, W. R.; *Methods Enzymol.* **1978**, *57*, 445.
13. Peterson, B. A.; Hansen, E. H.; Ruzlcka, J.; *Anal. Lett.* **1988**, *79***,** 649.
14. Gorus, F.; Schram, E.; *Clin. Chem.* **1979**, *25*, 512.
15. Lin, J. M.; Masaaki, Y.; *Anal. Chem.* **1999**, *71*, 1760.
16. Kamidate, T.; Maruya, M.; Tani, H.; *Anal. Sci*. **2009**, *25*, 1163.
17. Van Veldhoven, P. P.; Vanhove, G.; Assselberghs, S.; Eyssen, H. J.; Mannaerts, G. P.; *J. Biol. Chem.* **1992**, *267*, 20065.
18. Lowry, O. H.; Rosebrough, N. J.; Farr A. L.; Randall, R. J.; *J. Biol. Chem.* **1951**, *193*, 265.
19. Igarashi, S.; Hinze, W. L.; *Anal. Chem.* **1988**, *60*, 446.
20. Li, J.; Song, G. Y.; Jiang, L. L.; Zhang, W. J.; Feng, Z. S.; *Prog. Biochem. Biophys.* **2004,** *31*, 11.

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