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ARTICLE TYPE

A Novel Electrochemical Method to Determine α-Amylase Activity

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In this paper, we report a novel electrochemical method that can be developed as a biosensor for simple and direct determination of α-amylase activity. The method is based on the hydrolysis of maltopentaose, the substrate of the enzyme, which is immobilized on the surface of a gold electrode, and the induced charge changes of the substrate-modified electrode. Specifically, the substrate maltopentaose is immobilized onto gold electrode surface via a simple and direct immobilization technique that involves a one-step and site-specific attachment of unmodified maltopentaose to hydrazide-derivatized surface. So, by analyzing the electrochemical signal obtained from the electro-active molecule $[Ru(NH_3)_5Cl]^{2+}$ during the hydrolysis of maltopentaose, the determination of α-amylase activity is achieved. Under optimized conditions, α-amylase activity can be assayed with a detection limit of 0.022 U/mL. The biosensor exhibits a rapid response, good stability and anti-interference ability. Furthermore, the biosensor has also been successfully applied to detect α-amylase in human serum, which shows acceptable accuracy compared to the currently used clinical method. The proposed method in this work may also have potential application of α-amylase determination in practical blood, diagnostics and food production in the future.

1 Introduction

α-Amylase (E.C. 3.2.1.1) catalyzes the hydrolysis of α-1,4 glycosidic bonds present in starch, glycogen and other related carbohydrates[1]. It is included in plants, animals and microorganisms and has extensive applications in medicine, textiles, fermentation and food industry[2]. Blood α-amylase concentrations are normally low and fairly constant and they increase in acute pancreatitis and salivary gland inflammation. In clinical chemistry, the determination of serum or urine α-amylase activity for the diagnosis of acute pancreatitis is a widely used procedure and reveals the occlusion of pancreas[3]. Moreover, inhibition of α-amylase activity can reduce occurrence of type II diabetes and it is an important drug target for treatment of diabetes. Considering importance of α-amylase in diagnostics and disease therapy, it is highly required to measure α-amylase activity with high sensitivity and selectivity.

Some published methods, such as spectrophotometry, chromatography and immunological methods, have been applied to determine the catalytic activity of α-amylase. However, these methods, most of which are spectrophotometric, have the disadvantage that they are sensitive to turbidity and require the coloration of the test solution. In contrast, electrochemical measurements do not have these problems[4]. Moreover, electrochemical methods are most advancing for the rapid detection with lower costs, smaller equipment size as well as lower power requirements[5,6]. Besides, electrochemical biosensors are simple, accurate, low-cost and sensitive[7,8]. So α-amylase activity in human serum has been measured with an oligosaccharide dehydrogenase-modified graphite paste electrode containing benzoquinone[9]. Since the addition of α-glucosidase to the solution containing maltopentaose can give a current response related to amylase activity, electrochemical detection of α-amylase activity is achieved. Meanwhile, a flow injection-type biosensor has been developed in order to measure the correlation between stress conditions and salivary amylase level[10]. In addition, another electrochemical method for the assay of α-amylase activity has also been proposed based on the determination of α-amylase-generated maltose using a peroxide electrode equipped with glucose oxidase, α-glucosidase and mutarotase immobilized on a cellophane membrane[11].

In this paper, we report a novel electrochemical method to measure the activity of α-amylase by firstly immobilizing the substrate maltopentaose onto the surface of gold electrode and then analyzing the induced charge changes of the substrate-modified electrode due to the hydrolysis of maltopentaose by α-amylase. Therefore, a simple and direct way has been developed to assay α-amylase activity.

2 Experimental

2.1 Materials and chemicals

α-Amylases (EC.3.2.1.1), cysteamine, succinic anhydride, diisopropylcarbodiimide (DIC), N-hydroxysuccinimide (NHS), Dimethylformamide (DMF), 4,7,10-trioxa-1,13-tridecanediamine, hydrazine monohydrate, pentaamminechlororuthenium (III) chloride ($[Ru(NH_3)_5Cl]^{2+}$) and maltopentaose were purchased from Sigma-Aldrich. Pancreatic amylase ELISA kit was obtained from Shanghai Walan Biotechnology Co., Ltd (Shanghai, China). Other chemicals were of analytical grade. All buffers were prepared with double-distilled water, which was purified with a Milli-Q purification system (Branstead, USA) to a specific resistance of 18 MΩ cm.

2.2 Electrode preparation

The gold electrode (3.0 mm diameter) was firstly cleaned with piranha solution (concentrated H_2SO_4 : 30 % H_2O_2 = 3:1) for 5

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min to eliminate the adsorbed material and then rinsed with double-distilled water. After that, the electrode was polished carefully with 1, 0.3 and 0.05 μm alumina slurry, respectively. Residual alumina powder was removed by sonicating the electrode sequentially in both ethanol and double-distilled water. Afterward, the electrode was cleaned electrochemically to remove any remaining impurities in 0.5 M H_2SO_4. Finally, the electrode was dried by purging with nitrogen.

After being dried with nitrogen, the electrode was incubated firstly with 150 μL of cysteamine solution overnight and then with succinic anhydride (3%) for 3 h. After that, the electrode was washed with DMF and successively dipped in solutions of DIC (3%) and NHS (3%) both for 30 min and 4,7,10-trioxa-1,13-tridecanediamine (3%) for 3 h. To increase sensitivity of maltopentaose-coated electrode, the electrode was continuously incubated with solutions of succinic anhydride (3%) for 3 h, DIC (3%) and NHS (3%) for 30 min, and hydrazine monohydrate (3%) for 3 h. After washing with DMF, the electrode was immersed into a solution of maltopentaose (PBS containing 30% glycerol, 20 mM, pH 5.0) for 12 h. Finally, in order to avoid the nonspecific absorption, a solution of 1% Tween 20 was added to the electrode surface. Finally, the maltopentaose-modified electrode was dried by purging with nitrogen and then stored at 4 °C.

2.3 α-Amylase digestion of maltopentaose immobilized on the electrode

The surface reaction of the immobilized substrate maltopentaose catalyzed by α-amylase was carried out as follows. Firstly, 150 μL of α-amylase solution (PBS containing 0.1% Tween 20, pH 6.9) was employed firstly to pre-incubate at 37 ºC for 10 min. Then the electrode was immersed in the solution at 37 ºC for 9 min. Afterward, the electrode was thoroughly rinsed with DMF to terminate the reaction.

2.4 Electrochemical measurement

A three-electrode system consisting of the modified gold electrode, saturated calomel reference electrode (SCE) and platinum counter electrode was used for all the electrochemical measurements. All test solutions were thoroughly deoxygenated by bubbling high-purity nitrogen through the solution for at least 30 min. A stream of nitrogen was then blown gently across the surface of the solution in order to maintain the solution anaerobic throughout all the experiments.

Cyclic voltammetry (CV) and chronocoulometry (CC) were carried out on a CHI660D electrochemical analyzer (CH Instrument) in 20 mM HEPES (pH 6.0) containing 50 μM $[Ru(NH_3)_5Cl]^{2+}$. Cyclic voltammogramms were obtained over the potential scan range from 0 to -0.5 V at the scan rate of 100 mV/s. CC curves were recorded in the potential scan range from 0.2 to -0.5 V. Electrochemical impedance spectroscopy (EIS) was carried out on a CHI660C electrochemical analyzer (CH Instrument) in 0.1 M PBS (pH 7.2) containing 1 M KNO_3 and 5 mM $[Fe(CN)_6]^{3-/4-}$. EIS was performed by applying a bias potential of 0.224 V vs. SCE and 5 mV amplitude in the frequency range from 0.1 Hz to 100 kHz.

3 Results and discussions

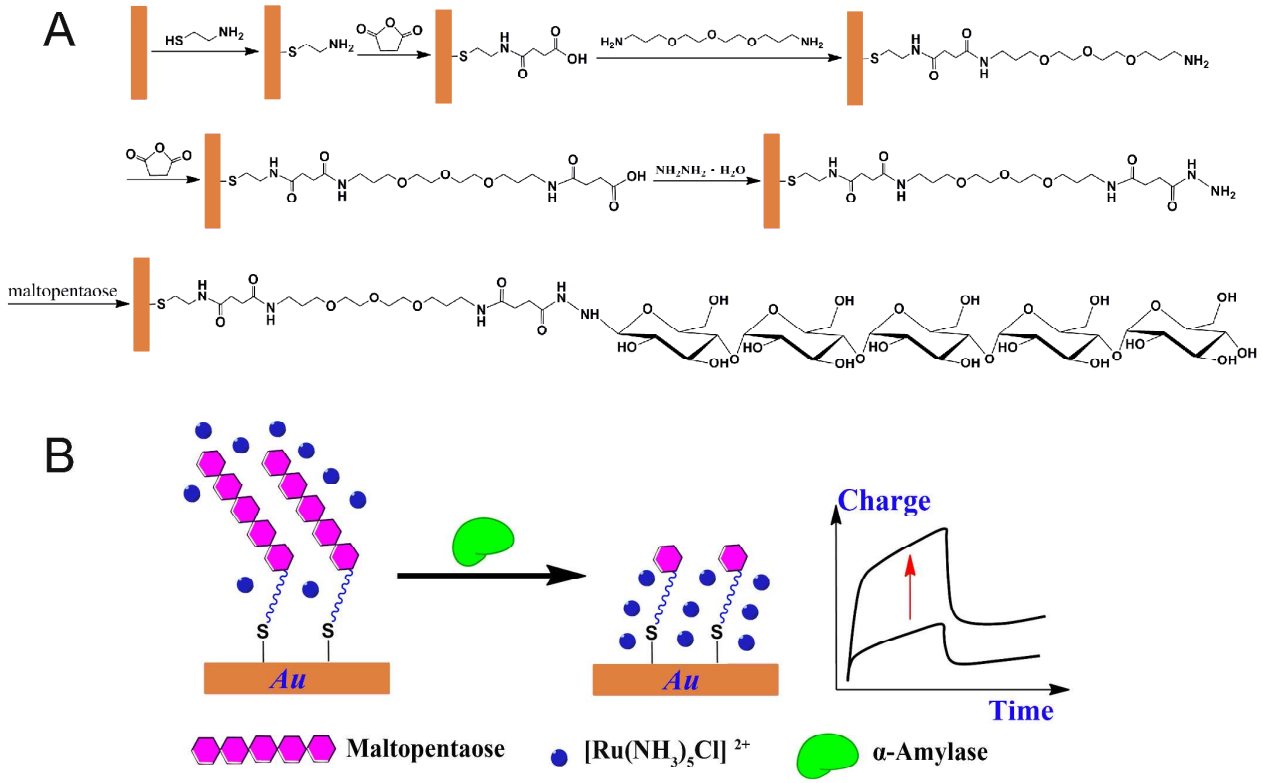

Fig. 1 (A) Preparation of maltopentaose modified gold electrode surface. (B) Schematic illustration of the mechanism to assay α-amylase activity with a simple and direct electrochemical method.

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The immobilization process of maltopentaose on gold electrode surface is shown in Fig. 1A, which makes it possible to directly hydrolyze the substrate maltopentaose on an electrode surface for the detection of α-amylase activity. The immobilization makes use of the strategy of site-specific and size-independent covalent attachment of unmodified sugar to proper surfaces[12,13], so immobilization of unmodified maltopentaose on hydrazide-derivatized surface can be achieved.

The mechanism of the proposed method for the detection of α-amylase activity is illustrated in Fig. 1B. With the immobilization of maltopentaose on the gold electrode surface, electroactive probe $[Ru(NH_3)_5Cl]^{2+}$ barely gets access to the electrode surface due to the long chain and plane structure of maltopentaose. However, after the electrode is incubated with α-amylase, maltopentaose will be cleaved. The specific cleavage of maltopentaose by the enzyme will decrease the density of the species on the electrode surface, resulting in a weak blocking effect against $[Ru(NH_3)_5Cl]^{2+}$ and an observed electrochemical signal. Therefore, a simple, rapid and easily operated electrochemical method for the assay of α-amylase activity can be proposed. Meanwhile, since the substrate maltopentaose modified gold electrode can be directly used for the detection, an electrochemical biosensor to assay α-amylase activity can be developed.

3.1 Characterization of the modified gold electrode

EIS is an efficient tool to study the interface properties of surface-modified electrode[14]. The reactions on the surface of gold electrode have been characterized with EIS. As shown in Fig. 2, no impedance can be observed on the bare gold electrode. After succinic anhydride has been immobilized again on the electrode, the interfacial electron resistance is increased evidently, ascribing to the covalent binding between the succinic anhydride and the long chain of 4,7,10-trioxa-1,13-tridecanediamine.

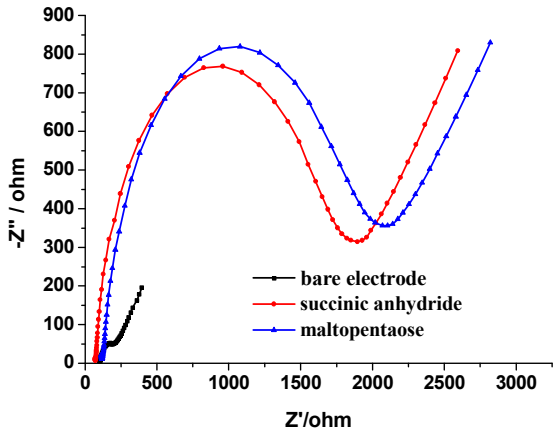

Fig. 2 Electrochemical impedance spectra obtained at the bare gold electrode, succinic anhydride modified electrode and maltopantaose modified electrode. The test solution is 0.1 M PBS buffer solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 1 M KNO_3 at pH 7.2.

Furthermore, after the further immobilization of substrate maltopentaose, more evidently increased interfacial electron resistance can be observed, as a result of the raising density of the electrode surface, which hinders electron transport between $Fe(CN)_6^{3-/4-}$ and the gold electrode.

3.2 Optimization of reaction condition

The effects of pH, temperature and time on the charge response for α-amylase activity have been investigated and the corresponding results are exhibited in Fig. S1. The influence of pH has been examined in the range from 5.4 to 8.9. The hydrolysis rate increases with the increase of pH values in the buffer solution and reaches a maximum at pH 6.9, which suggests that the activity of α-amylase maximizes (Fig. S1). Meanwhile, as shown in Fig. S2, reaction temperature has an obvious effect on the sensor. The investigation on the temperature-dependent activity shows that the reaction rate varies along with change of temperature. The activity of enzyme sharply increases at 37 ºC, and then decreases fast with increasing temperature, indicating the enzyme with optimized temperature at 37 ºC. Reaction time also has a great influence on the sensor (Fig. S3). With the prolong time, the current value increases, implying that maltopentaose are gradually hydrolyzed in the presence of α-amylase. The value almost keeps unchanged after 9 min, which suggests that maltopentaose have been totally hydrolyzed.

3.3 Electrochemical analysis of α-amylase by cyclic voltammetry

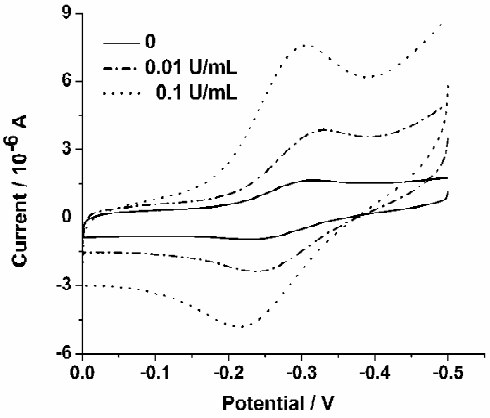

Fig. 3 Cyclic voltammograms obtained at the maltopentaose-modified gold electrode in 20 mM HEPES buffer (pH 6.0) containing 50 μM $[Ru(NH_3)_5Cl]^{2+}$ after incubation of the electrode with 0, 0.01 and 0.1 U/mL α-amylase, respectively.

CV is a valuable way to monitor the status of the modified electrode, so it has been employed to characterize the electrochemistry of the maltopentaose-modified gold electrode with probe $[Ru(NH_3)_5Cl]^{2+}$. The typical cyclic voltammograms before and after the electrode has been incubated with the test solution containing different concentration of α-amylase are

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given in Fig.3. Obviously, prior to the incubation of the electrode with α-amylase, a pair of peaks can be observed at the maltopentaose-modified electrode, but the peak current is weak, due to the blocking effect of the substrate maltopentaose on the positive charged electroactive probes blocked by. However, after the hydrolysis of maltopentaose by α-amylase (0.01 U/mL), a pair of obvious redox peaks can be observed and the value of peak currents is greatly increased. This indicates that the substrate maltopentaose on the electrode surface has been removed due to the enzymatic cleavage, thus $[Ru(NH_3)_5Cl]^{2+}$ can easily get access to the electrode surface to give electrochemical signals. Furthermore, the current value increases along with the raising α-amylase concentration from 0.01 to 0.1 U/mL, as a result of the hydrolysis of more substrate by the enzyme.

3.4 Quantitative detection of α-amylase by chronocoulometry

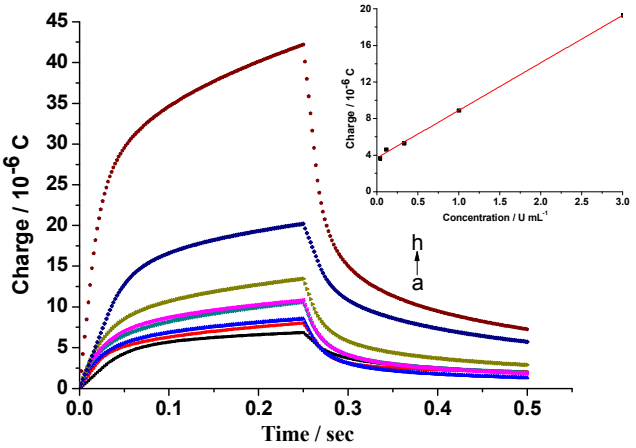

Fig. 4 CC curves for the assay of α-amylase activity at different concentrations. (a-h): 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1 and 3 U/mL. Inset shows a linear relationship between the CC current and the concentration of α-amylase ranging from 0.03 to 3 U/mL.

In order to more sensitively detect α-amylase activity, a much more accurate electrochemical technique CC has been further utilized for this study[15]. As shown in Fig. 4, the CC wave increases in response to the enzymatic activity over a range from 0 to 3 U/mL. So, in presence of more α-amylase, the amount of the substrate removed from the electrode surface is increased, deriving from the cleavage of more maltopentaose immobilized on the electrode surface. Consequently, with the addition of more α-amylase in the test solution, higher peaks are obtained as a result of more $[Ru(NH_3)_5Cl]^{2+}$ probes near to the electrode surface.

Furthermore, it also gives a linear relationship between the surface charge density of the electrode and the concentration of α-amylase changed from 0.03 to 3 U/mL and follows the regression equation of $Q = 3.69 + 5.20\ c$ (C, U/mL, R = 0.9984). The limit of detection (LOD) for α-amylase activity is estimated to be 0.022 U/mL (3 times signal-to-noise ratio), which is much lower than those in the previous reports[11,16]. Moreover, the detection range (0.03 to 3 U/mL) is much wider compared to those obtained by spectrophotometer technique[17] and colorimetric method[18]. A series of repetitive measurements with different concentration of α-amylase give a relative standard deviation within 10%, so the reproducibility of the detection can be also satisfactory.

3.4. Detection specificity of the biosensor

Table 1 Interference experiments using the proposed method for α-amylase detection

Interfering species	$Charge_{\alpha\text{-amylase + interfering species}}$ /$Charge_{\alpha\text{-amylase}}$
l-Cysteine	98.5%
Ascorbic acid	100%
Urea	95.48%

It is well known that some electroactive species in serum, such as urea, l-cysteine and ascorbic acid, may influence the performance of a biosensor[19]. Therefore, the anti-interference ability of the fabricated biosensor applied in α-amylase activity detection has been investigated. In this work, 5 mM urea (more than physiological levels), 0.1 mM l-Cysteine (more than physiological levels), and 0.1 mM ascorbic acid (around physiological levels) are injected into the reaction system, respectively. As seen from Table.1, these interferences don't have any obvious effect on the biosensor. The excellent anti-interference ability of the biosensor is attributed to the following two aspects. Interfering species are likely blocked by the substrates modified on the biosensor surface. Besides, the specific interaction between maltopentaose and α-amylase plays a major role in the process of measurement. So, the developed biosensor can exhibit good quality in anti-interference capability.

3.5. Stability of the biosensor

Table 2 Stability experiments of the developed biosensor for α-amylase detection

Time	$Charge_{\ store\ time}$ /$Charge_{\ control}$
15 days	98.85%
30 days	97.47%

The stability of the biosensor has been evaluated by recording the response with 0.3 U/mL of α-amylase concentration (Table.2). The biosensors have been stored under dry conditions at 4 ºC over 30 days. After 15 days, the biosensor retains about 98.85% of its original response. The response further decreases to 97.47% after 30 days. Good long-term stability of the biosensor can be attributed to the covalent conjunction between substrate maltopentaose and gold electrode surface.

3.6. α-Amylase detection in serum samples

Table 3 Results obtained from α-amylase detection in bovine and human serum

Sample	The present method (U/mL)	Pancreatic amylase ELISA kit (U/mL)	Normal (U/mL)
Bovine serum	0.098	0.102	0.069 - 0.113
Human serum	0.105	0.108	0.030 - 0.110

To evaluate the ability of the biosensor in practical analytical applications, the biosensor has been applied to detect α-amylase in fetal bovine and human serum samples. As seen in Table.3, the α-amylase concentration in a fetal bovine serum sample is determined to be 0.098 U/mL using the biosensor fabricated in this work, which is in agreement with the data 0.102 obtained through pancreatic amylase ELISA kit. The obtained data are also within the normal level changing from 0.069 to 0.113 U/mL[20] Meanwhile, the value of α-amylase obtained in human serum at 0.105 U/mL also agree well with the value 0.108 determined by

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pancreatic amylase ELISA kit, which is also within the normal range from 0.030 to 0.110 U/mL[21]. So, the comparable responses for α-amylase detection in two serum samples indicate that the proposed method is feasible for practical applications.

4. Conclusion

In summary, a simple and direct electrochemical method to assay the activity of α-amylase is proposed through immobilizing the unmodified substrate of the enzyme, maltopentaose, on gold electrode surface. Another advantage of this method is to keep away from immobilization of enzyme on electrode, so as to keep the high activity of α-amylase. The developed biosensor also shows excellent stability, high sensitivity, good anti-interference ability and fine specificity. The sensor can not only permit detection of α-amylase as low as 0.022 U/mL, but also be applied in determination of target α-amylase in serum sample. So, the proposed sensor may have potential applications for the α-amylase determination in practical blood, diagnostics and food production in the future.

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Notes and references

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