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α-Amylase immobilized on bulk acoustic-wave sensor by UV-curing coating

Deliang He, Yan Cai, Wanzhi Wei, Lihua Nie, Shouzhuo Yao*

College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, China

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Abstract

A new method for immobilization of α-amylase by UV-curing coating is proposed in this paper. The immobilization procedure of UV-curing coating on piezoelectric quartz crystal is simple and convenient, and causes less loss of enzymatic activity. The activity of the immobilized α-amylase is monitored by a technique based on bulk acoustic-wave (BAW) sensor. The frequency shift of BAW sensor can reflect the degree of hydrolysis of starch by the immobilized α-amylase. It is appropriate for the immobilized α-amylase to hydrolyze the soluble starch under pH 7.0 condition, which is similar to that of the free α-amylase. Kinetic parameters (the Michaelis constant, K_m, and the maximum initial rate V_{max}) of the enzymatic hydrolysis of starch by the immobilized α-amylase are estimated by using a linear method of Lineweaver–Burk plot. K_m=12.7 mg ml^{-1} and V_{max}=15.9 Hz min^{-1}. And the experimental results show that the immobilized α-amylase entrapped by the UV-curing coating retains adequate enzymatic activity and can be reused more than 50 times under certain experimental conditions.

Keywords: α-Amylase; Immobilization; UV-curing coating; Bulk acoustic-wave sensor

1. Introduction

Enzymes can be immobilized in a variety of water-soluble and water-insoluble matrices with little or no immediate loss of their catalytic activity [1]. Immobilization of soluble enzymes on or in organic or inorganic matrices permits researchers to add, remove, and reuse them at will. It is economical for industrial processors to substitute soluble enzymes with immobilized enzyme systems in some case. These are of interest, and importance, for theoretical reasons and industrial applications.

Many methods of enzyme immobilization have been reported in the literature, such as: adsorption [2], adsorption and cross-linking [3], cross-linking [4], ion-exchange resins [5], entrapment [6], microencapsulation [7], copolymerization [8], and covalent attachment [9]. Entrapment is an important immobilization method for enzymes. Bayhan immobilized α-chymotrypsin via physical entrapment within large, uniformly spherical, and thermally reversible poly(*N*-isopropylacrylamide) [poly(NIPAM)] beads [10]. Baran et al., [11] immobilized beta-galactosidase by entrapment in the bulk of the poly(2-hydroxyethylmethacrylate) (pHEMA) membranes, the storage stability of the enzyme was found to increase upon immobilization. Nakao et al. [12] used calcium alginate gel beads to entrap glucose oxidase as well as palladium particles to decompose hydrogen peroxide formed together with gluconic acid. Abdel-Naby et al. [13] immobilized alkaline protease from *Bacillus mycoides* on various carriers by different methods including: physical adsorption on hitoson, ionic binding on Amberlite IR-120, covalent binding on chitin, and entrapment in 2% cross-linked polyacrylamide. Ortega [14] immobilized β-glucosidase by entrapment in both calcium alginate and polyacrylamide gels. Centonze et al. [15] reported a glucose biosensor based on entrapment of glucose oxidase (GOD) in a poly(*o*-phenylenediamine) (PPD) film synthesized onto a conducting organic salt (COS) electrode. Besides, many materials were used for enzyme entrapment, such as: polymers, silicone rubber, silica gel, starch, etc.

α-Amylase (1,4-α-D-glucanglucanohydronase, EC 3.2.1.1) exists extensively in plants, animals and microorganisms. In the human body, it is present in fluids including serum, saliva, urine, etc. It is well known that α-amylase is applied widely in food industry, beer production, and other drink manufactures. But once used, α-amylase cannot retrieve easily from the reaction systems. So, it is very valuable to employ immobilized α-amylase to supercede free α-amylase. UV-curing coating was selected to immobilize α-amylase on bulk acoustic-wave (BAW) sensor by entrapment in this experiment. UV-curing coatings, which are generally formulated from the following components:

* Corresponding author. Tel.: +86-731-8822286; fax: +86-731-8824525.
E-mail address: szyao@mail.hunu.edu.cn (S. Yao)

prepolymer, monomer, photoinitiators and additives, cover many fields of applications [16–20]. These coatings, based on free-radical or cationic polymerization of unsaturated monomers, can solidify quickly after UV-light radiation in a few seconds. They will not pollute environment as a result of no solvents and cause less damage to the catalytic activity of enzyme.

Some published methods have been applied to determine the catalytic activity of α-amylase, such as spectrophotometry [21,22], fluorimetry [23], amperometry [24], electrophoresis [25,26], isoelectric focusing [27], chromatography [28] and immunological method [29]. α-amylase hydrolyzes internal α-1,4 linkage of starch (glucose residues in α-1,4 linkage) to yield maltose, maltotriose, and α-dextrin [30]. The viscosity of the starch solution has obvious change during the hydrolysis of starch by α-amylase. Therefore, the enzymatic hydrolysis of starch by immobilized α-amylase could be monitored by BAW sensor based on the viscosity change of the starch solution [31].

The BAW sensor has been extensively used as a kind of highly sensitive chemical and biological sensor in various fields, since it oscillated successfully in liquid phase in the 1980s [32–36]. The resonant frequency of the piezoelectric quartz crystal (PQC) changes with the viscosity and density change of the liquid, the following equation is effective under certain experimental conditions:

$$\Delta F = -F_s^{3/2} \left(\frac{\eta_L \rho_L}{\pi \mu_Q \rho_Q} \right)^{1/2} \quad (1)$$

where ΔF is the frequency shift of the crystal, F_s the resonant frequency, η_L the viscosity of the liquid, ρ_L the density of the liquid, μ_Q the shear modulus of the quartz crystal, and ρ_Q the density of the quartz crystal. Based on relationship 1, the BAW sensor can be used as a viscosity and density detector just like the technique presented in this paper. According to the response of BAW sensor, the enzymatic hydrolysis process can be monitored. The effect of pH on the enzymatic activity of α-amylase immobilized by UV-curing coating is studied by BAW sensor. And kinetic parameters (the Michaelis constant, K_m, and the maximum initial rate V_{max}) are estimated by using the Lineweaver–Burk plot [37,38]. Also, the reusing times of the immobilized α-amylase by UV-curing coating is studied by BAW sensing technique.

2. Experimental

2.1. Apparatus

The experimental assembly employed is shown in Fig. 1. A 9-MHz AT-cut crystal (JA-5 model, diameter 12 mm) with Au electrode (diameter 5.5 mm) was presented by the Peking Factory No. 707. One side of the crystal was positioned at the bottom of a well-type cell. The IC-TTL oscillating circuit made in Xiangtan Printed Circuit Factory of Hunan was supplied with 5 V by a d.c. voltage regulator (Model JWY-30B, Shijazhuan Electronic Factory No. 4). The detection cell was placed in a self-made air-bath chamber which was thermostated at 20±0.2°C. Frequency changes were measured with a digital counter (Model SS3341A, Shijazhuan Electronic Factory No. 4). The tested solution was stirred with a magnetic stirrer (Shanghai Electro-communication Instrumentation Factory). The UV-curing machine used for the immobilization of α-amylase was provided by the Machinery and Electric Factory of Hunan University. The working conditions were arranged in Table 1.

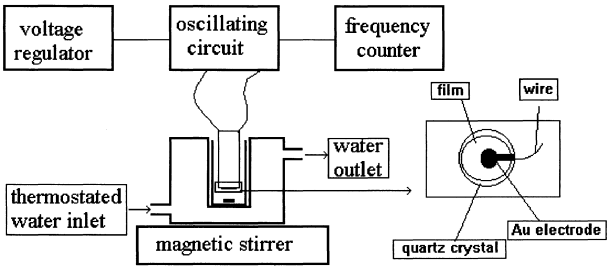

Fig. 1. Schematic representation of the experimental assembly.

2.2. Reagents and chemicals

α-Amylase (from *Bacillus subtilis*, 300 U mg^{-1}) was obtained from Shanghai Boao Biological Technical Company. One activity unit is the amount of amylase that liberates 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20°C. Soluble starch powder was the product of Peng Count Junle Chemical Factory. A series of starch solutions were prepared by dissolving starch in 0.1 mol l^{-1} phosphate buffer solution, then were stored in a refrigerator before use. The UV-curing coating was obtained from Weisheng High-Technical Company of Hunan University. The prepolymer (WS-6810-1) used in the UV-curing coating was *p*,*p*′-bisphenyl phenol A epoxide acrylic resin, its structural formula is shown in Fig. 2. All other chemicals were of analytical grade. Freshly doubly distilled water was used throughout.

2.3. Immobilization of α-amylase by the UV-curing coating

The Au plated piezoelectric quartz crystal (PQC) was cleaned with chloroform and acetone and dried in the air. The α-amylase powder and the UV-curable coating were mixed thoroughly according to the weight proportion of 3:7. Thereafter, 0.3 mg of the admixture was daubed on one side

Table 1
Working conditions of the UV-curing machine

Power of UV lamp (kW)	Radiation intensity (W cm^{-1})	Distance of two lamp (cm)	Solidification rate (m min^{-1})
3	80	10	8

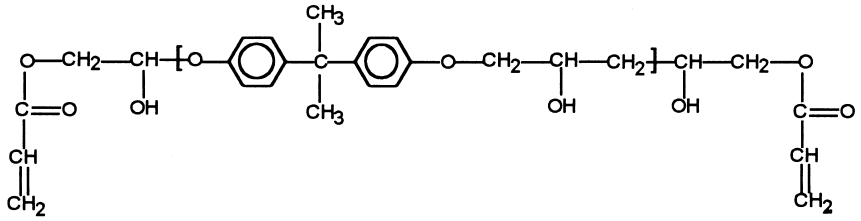

Fig. 2. Structural formula of *p*, *p*′-bisphenyl phenol A epoxide acrylic resin.

of the PQC, then the PQC with the admixture was rotated at a rate of 20 rpm for 30 min to spread the mixture uniformly on the PQC surface. After these processes, the PQC covered with a thin glass cup was put into the UV solidification operation machine. So a thin film of UV-curing coating including α-amylase was coated on the PQC surface. A series of BAW sensor with immobilized α-amylase within UV-curing coating were prepared.

2.4. Frequency shift measurements of the enzymatic hydrolysis of starch

The BAW sensor coated with a thin UV-curing coating film was steeped into 0.1 mol l^{-1} phosphate buffer solution (pH 6.9) for 5 min. Then, 1.0 ml starch solution was taken out from the stock and put into the detection cell and the starch solution was hydrolyzed by the immobilized α-amylase on one side of PQC. At the same time, the resonant frequency of BAW sensor was recorded. The frequency shifts with time were measured under different pH values and concentrations of starch solutions. The frequency shifts were also recorded while the BAW sensor was reused to hydrolyze the starch solutions with identical concentration. The frequency shifts were measured three times, and the mean values were calculated.

3. Results and discussion

3.1. Typical frequency shift curves in different starch solutions

The viscosity of the starch solution has obvious change when the immobilized α-amylase by UV-curing coating is used to hydrolyze the starch. This will result in the resonant frequency shift of the BAW sensor. The typical frequency shift curves of the enzymatic hydrolysis of starch by the immobilized α-amylase are shown in Fig. 3. The resonant frequency of PQC decreased with time and ΔF is diverse in different concentration starch solutions. The frequency change of BAW sensor means the viscosity of the starch solution has varied. That is to say that starch has been hydrolyzed partly by the immobilized α-amylase. So the response of BAW sensor can reflect the enzymatic hydrolysis of starch and the activity of the immobilized α-amylase.

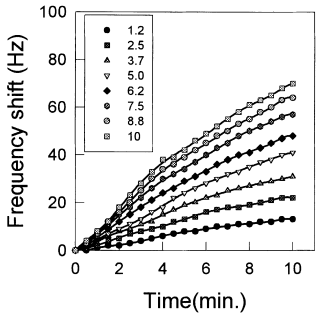

Fig. 3. Typical response curves of BAW sensor during the enzymatic hydrolysis of a series of starch solutions.

3.2. Effect of pH on the immobilized α-amylase

One mil1iliter each of 5.0 and 7.5 mg ml^{-1} starch solutions was hydrolyzed by the immobilized α-amylase on the BAW sensor, respectively. The pH of the starch solutions varied from 6.0 to 8.0. The frequency shift of 10 min ($\Delta F_1 = F_0 - F_1$, F_0 is the initial frequency of BAW sensor, F_1 the frequency of BAW sensor after 10 min) was recorded to describe the pH effect on the enzymatic activity of the immobilized α-amylase. When an enzyme is immobilized either within a matrix or on the surface of a carrier, the effect of pH on the enzymatic activity of the enzyme may change, depending on the nature of the carrier [39]. It is necessary to know the influence of pH on the activity of the immobilized α-amylase. The effect of pH on the activity of the immobilized α-amylase is shown in Fig. 4. It can be found that the maximum response (ΔF_1) appears when pH is ≈7.0. So, it is appropriate for the immobilized α-amylase

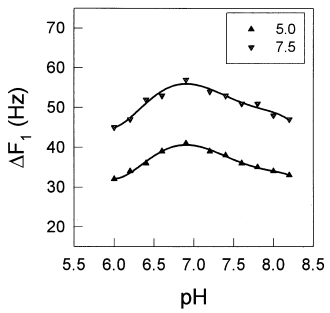

Fig. 4. The effect of pH on the activity of the immobilized α-amylase in 5.0 and 7.5 mg ml^{-1} starch solutions.

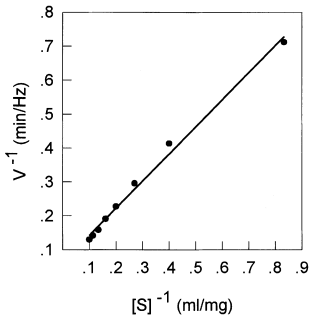

Fig. 5. The dependence of the reciprocal of enzymatic hydrolysis rate on the reciprocal of concentration of starch.

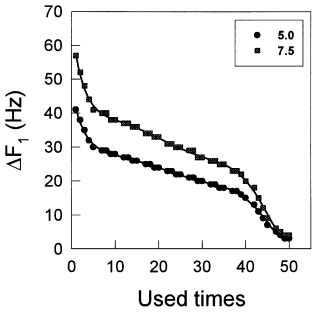

Fig. 6. The curves of the frequency shift (ΔF_1) of BAW sensor vs. the used times in 5.0 and 7.5 mg ml^{-1} starch solutions.

entrapped by UV-curing coating to hydrolyze the starch under the pH 7.0 condition, which is showing little difference to that of the free α-amylase. This implies that the nature of UV-curing coating has little influence on the enzymatic activity of α-amylase. So the succeeding experiments were carried out under pH 7.0.

3.3. Estimation of kinetic parameters of the immobilized α-amylase

The frequency shift of BAW sensor can mirror the viscosity change during the enzymatic hydrolysis of starch. The initial hydrolysis rate could be gained by linear fitting the response of BAW sensor within 3 min. Kinetic parameters of the enzymatic reaction can be estimated by the direct linear method of the Lineweaver–Burk plot [37,38] of the initial hydrolysis rates from BAW sensor:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (2)$$

where V and V_{max} are the initial hydrolysis rate and maximum hydrolysis rate in Hz min^{-1}, K_m the Michaelis constant in mg ml^{-1}, and [S] the starch concentration in mg ml^{-1}. Thus, a plot of the reciprocal of initial rate and the reciprocal of starch concentration for the immobilized α-amylase on PQC should give a straight line. Furthermore, the intercept gives $1/V_{max}$ and the slope is K_m/V_{max}, from which V_{max} and K_m can be calculated. In this work, the concentration range of starch was from 1.2 to 10.0 mg ml^{-1}. The experimental temperature was controlled at 20±0.2°C. The dependence of $1/V$ and 1/[S] is shown in Fig. 5, it can be seen that $1/V$ is linear to 1/[S]. By linear fitting according to Eq. (2) (n=8, r=0.982), the kinetic parameters are obtained, K_m is 12.7 mg ml^{-1} and V_{max} 15.9 Hz min^{-1}. The value of K_m is higher than that of free α-amylase (e.g. 6×10^{-4} g ml^{-1}) after α-amylase is entrapped into UV-curing coating.

3.4. Reusing times of the immobilized α-amylase

Two BAW sensor with immobilized α-amylase were reused to hydrolyze 1 ml of 5.0 and 7.5 mg ml^{-1} starch solutions (pH 7.0), respectively. Each was reused 50 times. The frequency shift of 10 min (ΔF_1) was recorded every time to check the activity loss of the immobilized α-amylase while it was reused. The α-amylase can be reused after it was immobilized, but the ability to hydrolyze the starch will decrease along with the increasing of the used times. The curves of the frequency shift (ΔF_1) and used times are shown in Fig. 6. The response decreased rapidly within six used times, while it had less variation from 8 to 35 used times. Afterwards, ΔF_1 declined increasingly. That is to say, the apparent activity of the immobilized α-amylase within the UV curing coating decrease while they were reused, this is because α-amylase may dissolve into the starch solution and the enzymatic activity may declined while the BAW sensor covered with UV-curing coating including α-amylase was reused. However, it is obvious that the α-amylase immobilized by UV-curing coating can be reused many times to hydrolyze the starch. The lifetime of the immobilized α-amylase can be estimated according to the response of BAW sensor when it is reused to hydrolyze the starch.

4. Conclusion

A new method for immobilization of α-amylase on PQC by UV-curing coating is proposed. It can be seen that the immobilization procedure of UV-curing coating is simple and convenient, and causes less enzymatic activity loss. Comparison between the two immobilization methods of α-amylase is shown in Table 2. Furthermore, compared with the spectrophotometric and fluorimetric method, the proposed method based on BAW sensor is convenient as no extra reagents such as chromogen or fluorescein is needed, and so it is simpler. Meanwhile it is fit for the continuous monitoring and study of the dynamics of the enzymatic hydrolysis process of starch. It is appropriate for the α-amylase immobilized with UV-curing coating to hydrolyze the starch under pH 7.0 condition, which is similar to that of free α-amylase used in this experiment. And kinetic parameters (K_m and V_{max}) are obtained by using the Lineweaver–Burk plot, K_m=12.7 mg ml^{-1} and V_{max}=15.9 Hz min^{-1}. The immobilized α-amylase can be reused to hydrolyze the 5.0

Table 2
Comparison of two immobilization methods of α-amylase

Method	Main materials	Immobilization conditions	Procedure	Industrialization	Reference
Cross-linking	APTES[a], TEA[b], IPD[c]	Low temperature and long reaction time	Three step, intricate	Difficult to achieve	[31]
Entrapment	UV-curing coating	Room temperature and short operating time	One step, simple and convenient	Easy to bring into effect	This paper

[a] Aminopropyltriethoxysilane.
[b] Triethylamine.
[c] Isophthaloyl dichloride.

and 7.5 mg ml^{-1} starch solutions for 50 times at a operation of 10 min. It could be prognosticated that the immobilized α-amylase by UV-curing coating could apply for industrial purpose, UV-curing coating could be used to immobilize some other enzyme, and some other enzymes could be studied after immobilized on PQC.

Acknowledgements

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