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Monitoring the Inhibitive Effect of the Static Magnetic Field on the Activity of Lysozyme with Acoustic Wave Impedance Analysis Technique

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The inhibitive effect of static magnetic field on the activity of lysozyme was studied using acoustic wave impedance analysis technique. Equivalent circuit parameters of piezoelectric quartz crystal (PQC) were obtained and discussed. The results showed that the activity of lysozyme was inhibited due to the effect of static magnetic field and the inhibitive effect becomes greater with an increase in magnetization time or magnetic field intensity. According to the response characteristics of motional resistance change (ΔR_1), which is related to the change in the bacterial number, a quantitative response model reflecting the activity of lysozyme was theoretically derived. By fitting ΔR_1 versus time curves under a specific magnetic field intensity but different magnetic time to the model, the relationship between K_1 reflecting the activity of lysozyme and magnetic time t_m was established. Based on the relationship, a new impedance response model that indicates the inhibitive influence of the magnetization time on the activity of lysozyme was derived as follows: $\Delta R_1 = R_0\{\{K_4\{\exp[K_0 \exp(-0.26t_m)]t - 1\} + 1\}^{1/2} - 1\}$. Similarly, another response model that indicates the effect of magnetic field intensity was derived as follows: $\Delta R_1 = R_0\{\{K_4\{\exp(K_0 \exp(-5.17B)t) - 1\} + 1\}^{1/2} - 1\}$.

INTRODUCTION

With increasing public interest in the possible impact of magnetic field on man and the environment, various biological effects of magnetic field have become the subjects of many investigations in terms of biomagnetics[1–3] and a great number of papers have appeared which concern almost all biological systems including the simple cell,[4,5] microorganisms,[6] lower animals[7] and man.[8] So far the magnetic technique has also been used to protect against subsequent hypoxia insult[9] and applied to separate erythrocytes from the whole blood utilizing the paramagnetic properties of hemoglobin in erythrocytes.[10]

The effect of magnetic field on enzymes is one of the most important aspects of biomagnetics because the enzyme is one kind of special protein having biological activity. The effect of magnetic fields on living organisms acts through DNA, RNA and enzymes mostly *in vivo*. Therefore, it is significant to study the effect of magnetic fields on enzymes. To our best knowledge, there are few qualitative analyses of the effect of magnetic fields on the activity of enzymes and fewer qualitative studies. This paper is aimed at reporting this aspect.

Acoustic wave impedance analysis technique is a practical method for studying the quartz crystal resonance and provides multidimensional information reflecting some physical-chemical properties of the investigated system.[11,12] For an intact quartz crystal, acoustic wave impedance analysis has been based on the Butterworth-Van Dyke (BVD) equivalent electrical circuit model (Figure 1), which is composed of a motional arm and a static arm in parallel. The motional arm contains three equivalent circuit elements in series, namely motional resistance (R_m), motional inductance (L_m) and motional capacitance (C_m), while the static arm only contains the static capacitance (C_0). All of four equivalent circuit parameters are of distinct physical meanings.[11–13] Acoustic wave impedance analysis technique has been successfully applied to the field of life sciences,

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including hemorheological characteristics,[14,15] detection of an enzyme and its activity[16,17] and monitoring of the cross-linking process.[18]

Lysozyme is a typical globular protein and its three-dimension structure is well known. The determination of lysozyme is very important in auxiliary clinical diagnosis and toxicological experiments. Accordingly, lysozyme was used here as a model enzyme to quantitatively analyse the inhibitive effect of static magnetic field on the activity of enzyme by acoustic wave impedance analysis technique. The study was based on the concept that *Microccus lysodeiteicus* (*M. lysodeiteicus*) was hydrolyzed due to the catalysis of lysozyme, thus the physical and chemical properties of the bacterial solution would change accordingly, which would cause the variations in the equivalent circuit parameters of PQC. The change of these impedance parameters could reflect the change of lysozyme activity. Thus, the inhibitive effect of static magnetic field on the activity of lysozyme could be studied by monitoring the change in these parameters.

MATERIALS AND METHODS

Reagents and Solutions

Phosphate buffer solution (PBS, pH 6.35) was prepared by dissolving 4.72 g of NaH_2PO_4 and 0.905 g of $K_2HPO_4{\cdot}3H_2O$ in 1000 ml of doubly distilled water.

Lysozyme (EC 3.2.1.17, 20,000 U/mg) was purchased from Sigma Chemical Corporation. Stock lysozyme solution (2 mg/ml) was prepared by dissolving in PBS, and stored at 0°C before use.

All reagents used were of analytical grade. Doubly distilled and sterilized water was used throughout.

Microorganism

The composition of the medium for *M. lysodeiteicus* was beef extract, 5.0 g; peptone, 10.0 g; sodium chloride, 2.0 g; agar, 8.0 g; and doubly distilled water, 1000 ml. After the medium was sterilized by autoclaving at 121°C for 30 min, the agar slants were prepared.

M. lysodeiteicus was purchased from Central South University (Changsha, China). The bacterium was inoculated into an agar slant and incubated at 37°C for 18 h. Then PBS at 37°C was used to rinse the colony and yield the suspension. The suspension was stored at 4°C before use.

Materials and Instrumentation

The AT-cut 9 MHz piezoelectric quartz crystal (12.5 mm in diameter) with a gold electrode on each side (6 mm in diameter) was purchased from State-run 707 factory (Beijing, China). To ensure the cleanliness of the gold-coated electrode, the surface of the gold electrode was first treated with $H_2SO_4 + H_2O_2$ (V/V 3:1) for 5 min, then cleaned with water, acetone, water sequentially. Before use, the electrode was sterilized by autoclaving at 121°C for 30 min.

The experimental setup is shown in Figure 1. The system consisted of a HP4192A LF network spectrum impedance Analyzer with one side connected to the terminal contacting liquid of PQC sensor, while the other side was connected with a personal computer in which a user program was written in Visual Basic 6.0 to control the analyzer and to acquire admittance data. Ring magnet (i.d., 7.5 cm; o.d., 15 cm; thickness, 3.5 cm) was purchased from the center for magnet production (Changsha, China). The magnetic field intensity acting on the detection system was measured with a CT 5 Hoare Gaussmeter.

Procedures

A bacterial suspension (2 ml) was injected into a detection cell, and then the PQC sensor was immersed. The detection cell was sealed with a rubber plug and the temperature was controlled

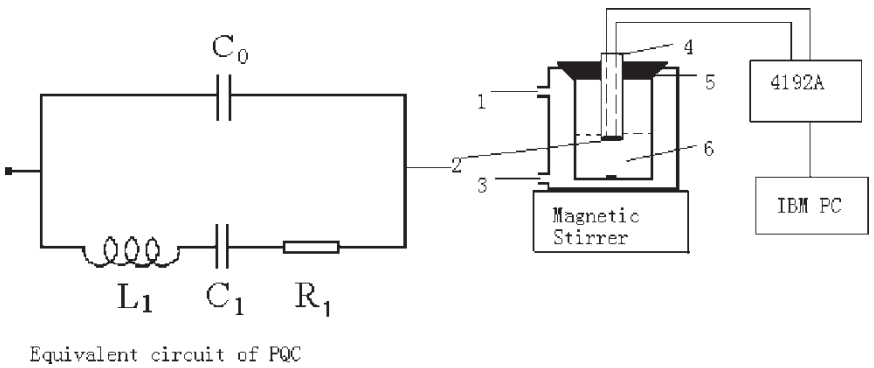

FIGURE 1 Schematic representation and equivalent circuit of the acoustic wave impedance analyzer. (1) Thermostatic water outlet; (2) Quartz crystal sensor; (3) Thermostatic water inlet; (4) PVC tube; (5) rubber stopper; (6) Detection solution; C_0—static capacitance; L_1—motional inductance; C_1—motional capacitance; R_1—motional resistance.

at 37 ± 0.1°C by a thermostatic water-jacket. After a stable frequency was reached, 50 μl of 2 mg/ml lysozyme solution at 37°C was added. The cell wall of *M. lysodeiteicus* began to dissolve and the impedance parameters began to change with time. The variations of impedance parameters were real time monitored by 4192A Impedance Analyzer.

RESULTS AND DISCUSSION

The Response Theory of Acoustic Wave Impedance Analysis

When the acoustic wave sensor is in gas, the Sauerbrey equation[19] shows a linear relationship between the mass change (Δm) of the piezoelectric quartz crystal (PQC) and the frequency shift (Δf_m) for the rigid and thin film loading;

$$\Delta f_m = -\frac{2f_0^2}{(\rho_Q \mu_Q)^{1/2}} \times \frac{\Delta m}{A} \quad (1)$$

where f_0 is the fundamental resonant frequency of PQC, ρ_Q and ρ_Q are the density and the shear modulus of quartz, respectively and A is the area of the quartz plate.

In liquid, the physical and chemical properties of the solution can also cause the frequency to change.[13] It has been pointed out by Kanazawa[20] that the effect of the viscosity and density of the liquid on the resonant frequency can be expressed as follows:

$$\Delta f_L = -\frac{f_0^{3/2}(\rho_L \eta_L)^{1/2}}{(\pi \rho_Q \mu_Q)^{1/2}} \quad (2)$$

where ρ_L and η_L are the viscosity and density of the liquid, respectively.

Then, the total frequency shift (Δf) is the sum of Δf_m and Δf_L:

$$\Delta f = \Delta f_m + \Delta f_L \quad (3)$$

Motional resistance R_1 corresponds to the loss in mechanical energy mainly dissipated to the surrounding medium and quartz interior. For the monitoring process of lysozyme activity,

$$R_1 = \frac{(2\pi f_0 \rho_L \eta_L)^{1/2} A}{\kappa^2} \quad (4)$$

where κ represents the electromechanical coupling factor.

In addition, Static capacity (C_0) originates from the two parallel plate metal electrodes in the dielectric property of the quartz. In a conductivity liquid, C_0 also corresponds to the capacity and structure of the electrical double layer at the charged interface[11]

$$C_0 = \frac{K_0 A \varepsilon}{e} \quad (5)$$

where K_0 is the permittivity of space, e is the thickness of the quartz crystal, and ε is the dielectric constant of the quartz.

If Δf is dominated by the net changes in the viscosity and density of the liquid, as for a presently used 9 MHz piezoelectric quartz crystal, the ratio of Δf and ΔR_1 should[12] be 9.45 Hz Ω^{-1}. In fact, $\Delta f/\Delta R_1$ is often greater than 9.45, because measured value of Δf may contain the contributions of both mass and the viscosity–density effect.

The Typical Response Curves of Impedance Parameters without the Effect of Magnetic Fields

The typical response curves for the impedance parameters (Δf, ΔR_1 and ΔC_0) during the bacteriolytic process without the effect of magnetic fields are shown in Figure 2 (a_1, a_2 and a_3). It can be seen that the addition of lysozyme led to a decrease in R_1 but an abrupt increase in f and a slow change in C_0. As shown in Equations (3) and (4), the changes in f and R_1 indicate the variations of the viscosity and density of the bacterial solution during the bacteriolytic process. It is interesting to note that the ΔR_1 curve shows a negative exponential attenuation trend. The trend can be expected from the point of the bacterial decay. Owing to the catalysis of lysozyme, the bacterial cell walls are ruptured, and thus the number of living cells decreases, which causes decreases in the viscosity and density of the bacterial

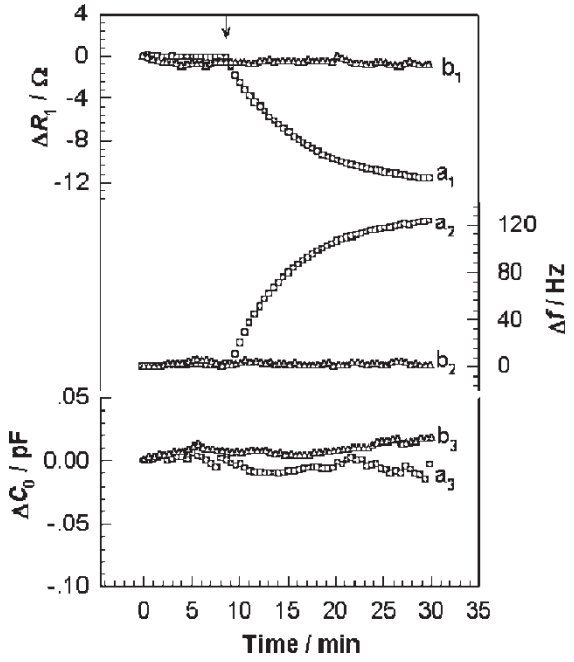

FIGURE 2 Time courses of simultaneous responses of ΔR_1, Δf and ΔC_0 during the bacteriolytic process without the effect of static magnetic field on the lysozyme. (1) The arrow indicates the time of lysozyme addition. (2) The initial concentration of the bacterial solution is 4.8×10^8 cells/ml, and the concentration of lysozyme in the detection system is 50 μg/ml.

solution. As a result the motional resistance R_1 decreases and resonant frequency f of PQC increases. The slope of the response curves of ΔR_1 and Δf versus time correspond to the rate of bacteriolysis and the signal sizes of the two parameters correspond to the activity of lysozyme. Here, the change of motional resistance (ΔR_1) was used to analysis the inhibitive effect of static magnetic field on the activity of lysozyme.

From Figure 2, an increase of ca. 124 Hz in Δf and a decrease of ca. 12 Ω in ΔR_1 can be obtained. In the current experiments, the ratio between Δf and ΔR_1 is estimated to be ca. 10.3 $Hz \cdot \Omega^{-1}$ and very close to the theoretical value 9.45 $Hz \cdot \Omega^{-1}$. If the bacterial cells and lysozyme are absorbed on the PQC surface during the bacteriolytic process, Δf will change much more, and the ratio between Δf and ΔR_1 will be much more than 9.45 $Hz \cdot \Omega^{-1}$. So the ratio of 10.3 $Hz \cdot \Omega^{-1}$ in this study showed that the contribution to Δf mainly originates from the decrease in the viscosity and density, and has hardly any mass effect. That is to say, no lysozyme or bacterial solution adheres onto the PQC surface. Therefore, the concentration of the lysozyme does not change and the bacteriolysis rate is not affected by the gold electrode of PQC.

From curve a_3 in Figure 2, it can be seen that ΔC_0 keeps a constant value basically during the bacteriolytic process. This result shows that the dielectric constant of the crystal quartz is almost unchanged.

In order to examine the effect of the background, the bacterial solution, in which no lysozyme was added, was monitored by the same technique. The impedance response curves are shown in Figure 2 (curve b_1, b_2 and b_3). It can be seen that the impedance parameters are not obviously changed. This indicates that neither obvious growth nor obvious spontaneous lytic behavior of the bacteria occurred during the detection process.

Effect of the Magnetization Time on the Activity of the Lysozyme

The ΔR_1 response curves in Figure 3 show the effect of different magnetization times on the activity of lysozyme under a magnetic field intensity of 0.2 T. Under different conditions, ΔR_1 curves display the similar changing trend. As mentioned before, the trend is due to the decrease in the viscosity and density of the solution during the bacteriolytic process. However, we could still investigate the difference between these response curves from two aspects. First, the decreased slope of ΔR_1 was different. Compared with the response curve without the effect of magnetic field (curve 1), ΔR_1 decreases more slowly with a longer magnetization time. This result demonstrates that the rate of bacteriolysis of lysozyme decreased due to the inhibitive effect of the static magnetic field and

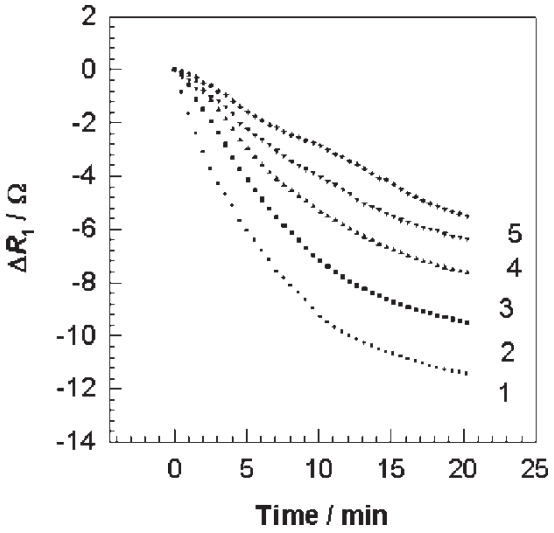

FIGURE 3 ΔR_1 response curves of the effect of magnetization time on the activity of lysozyme under a magnetic field intensity of 0.2T. The magnetization time: (1) 0 h; (2) 2 h; (3) 4 h; (4) 6 h; (5) 8 h. The initial concentration of the bacterial solution was 4.8×10^8 cells/ml.

the effect is greater with a longer magnetization time. Secondly, the signal size of the ΔR_1 response (ΔR_{max}) is different. A greater ΔR_{max} value is obtained when no magnetic field acted on the lysozyme. The signal size of ΔR_1 decreased more obviously with a longer magnetization time. This result indicates that the activity of lysozyme is inhibited by the effect of a magnetic field and the inhibitory extend is larger with a longer magnetization time.

Effect of the Intensity of Magnetic Field on the Activity of Lysozyme

The ΔR_1 response curves in Figure 4 exhibited the effect of different intensities of magnetic field on the activity of lysozyme with the magnetization time of 6 h. When the magnetization time is constant, the signal size of ΔR_1 and the slop of the response curves are smaller with a higher intensity of magnetic field. This may be because the three-dimensional structure of lysozyme is affected by the magnetic field. Thus part of the activity of lysozyme is lost or inhibited, and the effect is greater with a higher magnetic field intensity. So the variations of viscosity and density of solution are smaller with a higher magnetic field intensity, leading to a smaller signal size of ΔR_1 and the slop of the response curves. This phenomenon is reproducible through multiple experiments.

Establishment of Impedance Response Model

The rate of lysis is related to the number of bacterial cells and the amount of lysozyme. In addition, Schleif *et al.*,[21] also pointed out that the bacteriolytic process also includes spontaneous lytic behavior of

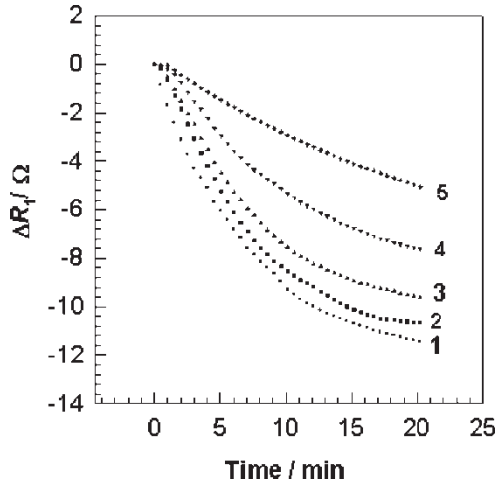

FIGURE 4 ΔR_1 response curves of the effect of different intensity of magnetic field on the activity of lysozyme with the magnetization time of 6 h. The intensity of magnetic field: (1) 0T; (2) 0.05T; (3) 0.1T; (4) 0.2T; (5) 0.4T. The initial concentration of the bacterial solution was 4.8×10^8 cells/ml.

the bacteria. Thus, the rate of lysis rate can be described as follows:

$$\frac{dN}{dt} = -K_1 N - K_2 N \tag{6}$$

where N (cell·ml^{-1}) is the number of bacterial cells; t (min) is the bacteriolytic time. K_1 (min^{-1}) and K_2 (min^{-1}) are the catalytic rate of lysozyme and spontaneous lytic rate of cells, respectively.

Equation (6) can be rewritten as:

$$\frac{dN}{N} = -(K_1 + K_2)dt \tag{7}$$

Equation (7) can be integrated to:

$$\ln\frac{N}{N_0} = -(K_1 + K_2)t \tag{8}$$

where N_0 (cell·ml^{-1}) is the initial number of bacterial cells. Equation (8) can also be rewritten as:

$$N = N_0 \exp[-(K_1 + K_2)t] \tag{9}$$

So the decrease in the bacterial cell number (ΔN) can be expressed as:

$$\Delta N = N_0 \exp[-(K_1 + K_2)t] - 1 \tag{10}$$

N is proportional to the gross of the compositions which constitute bacteria, while the latter is proportional to the viscosity and density ($\rho\eta$) of the bacterial solution. Therefore, ΔN is proportional to the variations of the viscosity and density of the bacterial solution:

$$\Delta N = K_3(\rho\eta - \rho_0\eta_0) \tag{11}$$

where K_3 is a constant reflecting the relationship between N and ($\rho\eta$); ρ_0 and η_0 are the initial density and viscosity of the bacterial solution, respectively.

From Equations (10) and (11), $\rho\eta$ can be expressed as:

$$\rho\eta = \frac{N_0}{K_3}\{\exp[-(K_1 + K_2)t] - 1\} + \rho_0\eta_0 \tag{12}$$

For an identical quartz crystal, Equation (13) can be obtained from Equation (4):

$$\frac{R_1}{R_0} = \left(\frac{\rho\eta}{\rho_0\eta_0}\right)^{1/2} \tag{13}$$

where R_1 and R_0 are the motional resistance at t time and zero time, respectively. The absolute values of R_1 and R_0 are also related to the test system.

Combining Equations (12) and (13), Equation (14) was obtained as:

$$R_1 = R_0\left\{\frac{N_0}{K_3\rho_0\eta_0}\{\exp[-(K_1+K_2)t]-1\}+1\right\}^{1/2}$$
$$= R_0\{K_4\{\exp[-(K_1+K_2)t]-1\}+1\}^{1/2} \tag{14}$$

where K_4 is a constant related to the N_0 and ($\rho_0\eta_0$) of the bacterial solution.

Thus the variation of motional resistance can be expressed as

$$\Delta R_1 = R_0\left\{\{K_4\{\exp[-(K_1+K_2)t]-1\}+1\}^{1/2}-1\right\} \tag{15}$$

From the lines b_1, b_2 and b_3 of Figure 2, it can be seen that no obvious spontaneous lytic behavior of the bacteria happened during the monitoring process. So Equation (15) can be substituted as:

$$\Delta R_1 = R_0\left\{\{K_4\{\exp(-K_1 t)-1\}+1\}^{1/2}-1\right\} \tag{16}$$

Equation (16) reveals the relationship between ΔR_1 response and K_1, K_4. Thus, an impedance response model reflecting the change in the activity of lysozyme is obtained. Taking K_4 and K_1 as estimation parameters, ΔR_1 response were fitted by using the nonlinear fitting program embedded in Sigmaplot Scientific Software Version 2.0. The relative sum (q_r) of Residual Square is used to reflect the validity of the fitting, and defined as:

$$q_r = \frac{\sum_1^n (\Delta R_{fit} - \Delta R_{exp})^2}{\sum_1^n \Delta R_{exp}^2} \tag{17}$$

where ΔR_{fit} and ΔR_{exp} denote the motional resistance change fitted and experimentally obtained, respectively; n is the number of the response signal points.

By fitting the experimentally obtained values of ΔR_1 in Figure 3 to the derived model, the fitted ones of ΔR_1 can be obtained. The fitting parameters are shown in Table I. It can be seen that catalytic rate of lysozyme, K_1, became smaller with longer magnetization time. As mentioned above, K_4 is related to N_0 and ($\rho_0\eta_0$) of the bacterial solution.

TABLE I Parameters obtained by fitting according to Equation (16) ($f_0 = 8.989$ MHz, $B = 0.2$T)

Magnetization time/h	K_1	K_4	q_r
0	0.5897	1.2293	7.96×10^{-4}
2	0.3363	1.2841	5.63×10^{-3}
4	0.1909	1.1579	7.85×10^{-4}
6	0.1259	1.0575	4.51×10^{-4}
8	0.0915	1.0847	2.04×10^{-4}

In our experiments the volume and compositions of the bacterial solution were almost kept the same. So it can be seen from Table I that K_4 changes little under different monitoring conditions. The satisfactory value of q_r shows that the ΔR_1 *vs* time curves can be used to reflect the bacteriolytic process, and this response model can reasonably describe the kinetic characteristics of the process.

Similarly, by fitting the experimental values of ΔR_1 in Figure 4 to the response model, the values of K_1 and K_4 affected by different intensities of magnetic field were obtained. The kinetic parameters are shown in Table II.

To study the effect of the static magnetic field on the activity of lysozyme in details, the curve of the K_1 *vs* magnetization time was plotted (see Figure 5). The curve of K_1 *vs* the intensity magnetic field is shown in Figure 6.

From Figure 5, it is apparent that kinetic parameter K_1 changes regularly with an increase in magnetization time. An exponential relationship exists between K_1 and the magnetization time. The regression equations were obtained as follows:

$$K_1 = K_0 \exp(-0.26 t_m) \tag{18}$$

where K_0 is catalytic rate of lysozyme without the effect of the magnetic field, K_1 is catalytic rate of lysozyme with the effect of magnetic field, t_m is the magnetization time.

From Equations (16) and (18), the following equation can be readily obtained:

$$\Delta R_1 = R_0 \left\{ \left\{ K_4 \left\{ \exp\left[K_0 \exp(-0.26 t_m) \right] t - 1 \right\} + 1 \right\}^{1/2} - 1 \right\} \tag{19}$$

This equation shows the relationship between the magnetization time and the ΔR_1 response, and it also reflects the inhibitive effect of the 0.2T magnetic field

TABLE II Parameters obtained by fitting according to Equation (16) ($f_0 = 8.989$ MHz, $t_m = 6$ h)

Magnetic field/T	K_1	K_4	q_r
0.00	0.5897	1.2293	7.96×10^{-4}
0.05	0.4877	1.4013	1.72×10^{-3}
0.10	0.3682	1.3138	5.62×10^{-3}
0.20	0.1911	1.1586	8.16×10^{-4}
0.40	0.0837	1.0612	2.83×10^{-4}

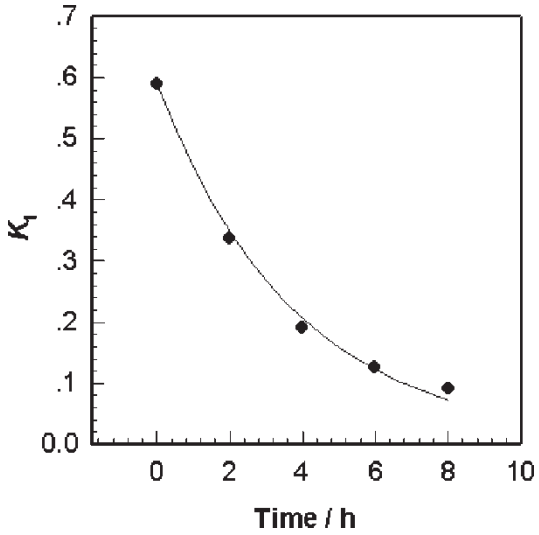

FIGURE 5 Relationship between K_1 and the magnetization time. The points are the experimental data. The line is the fitted data. The intensity of the magnetic field is 0.2T. The initial concentration of the bacterial solution was 4.8×10^8 cells/ml.

on the activity of lysozyme. The value of the magnetization time (t_m) can be estimated by using the measured ΔR_1 response according to Equation (19). Moreover, Equation (19) can be used to predict the ΔR_1 response from the known magnetization time. In other words, the activity of lysozyme can be predicted.

From Figure 6, it can be also seen that an exponential relationship exists between K_1 and the intensity of the magnetic field. The regression equation obtained was as follows:

$$K_1 = K_0 \exp(-5.17 B) \tag{20}$$

where B is the intensity of magnetic field.

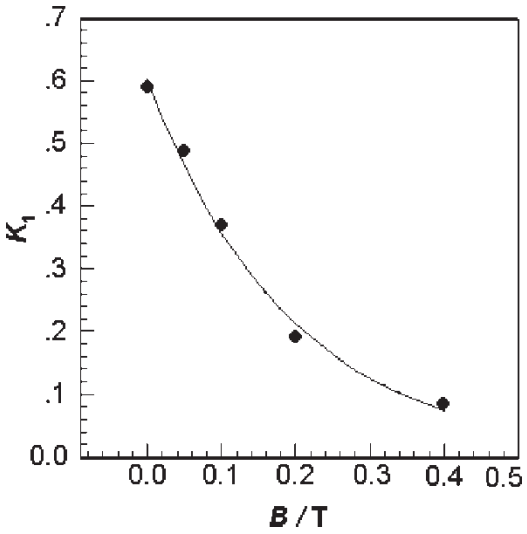

FIGURE 6 Relationship between K_1 and the intensity of magnetic field. The points are the experimental data and the line is the fitted data. The magnetization time was 6 h. The initial concentration of the bacterial solution was 4.8×10^8 cells/ml.

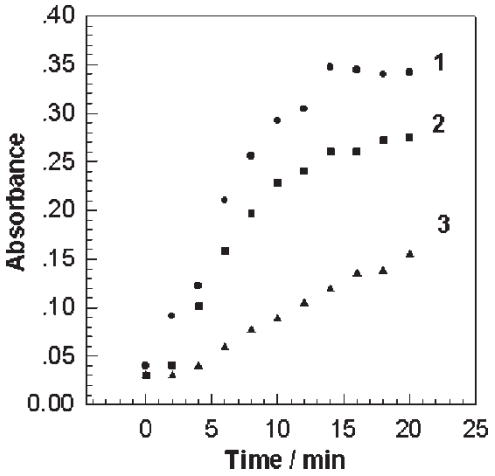

FIGURE 7 Response curves of the effect of a static magnetic field on the activity of lysozyme with a magnetization time 6 h by the turbidimetric method. Line 1, the intensity of the magnetic field is 0T. Line 2, the intensity of the magnetic field is 0.1T. Line 3, the intensity of the magnetic field is 0.4T. The initial concentration of the bacterial solution was 4.8×10^8 cells/ml.

From Equations (16) and (20), the following equation can be obtained:

$$\Delta R_1 = R_0\left\{\left\{K_4\left\{\exp(K_0\exp(-5.17B)t)-1\right\}+1\right\}^{1/2}-1\right\} \quad (21)$$

This equation shows the relationship between the intensity of the magnetic field and the ΔR_1 response, and it reflects the inhibitive effect of different intensities of the magnetic field on the activity of lysozyme. The value of B can be estimated by using the measured ΔR_1 response according to Equation (21). Moreover, Equation (21) can be used to predict the ΔR_1 response with known B value: in other words, the activity of lysozyme can also be predicted.

Comparison of the Proposed Method with the Traditional Turbidimetric Method

The proposed method was compared with the traditional turbidimetric method. The kinetics of reactivation of lysozyme was monitored by removing 500 μl samples from the detection solution at specific time intervals and quenching the reaction with 25 μl of 0.5 mol/l iodoacetic acid. The detection method is similar that previously described.[22] The absorbance versus time curves are shown in Figure 7. Comparing Figure 7 with the curves 1, 3, 5 in Figure 4, it can be seen that the acoustic wave impedance technique agreed well with the turbidimetric method. However, the new proposed method can avoid cumbersome operations and provide real-time and multidimensional response information (such as ΔR_1, Δf and ΔC_0) reflecting the variation of physical and chemical properties in solution during the monitoring process. In addition, fewer samples are needed and the concentration range of the bacterium, which can be used, is much wider.

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