SHORT COMMUNICATIONS

Effect of D-allethrin on proteinase activity in the midgut of the spider *Pardosa pseudoannulata* detected by bulk acoustic wave impedance technique*

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Abstract The piezoelectric bulk acoustic wave (BAW) impedance analysis method was employed to monitor *in situ* the proteinase-catalyzed hydrolyzation of casein and the effect of pesticide D-allethrin on the proteinase activity in the midgut of the spider *Pardosa pseudoannulata*, and the results obtained were verified by ultraviolet spectroscopy. The hydrolyzation was significantly increased in the presence of low-dose D-allethrin, whereas significantly inhibited in the presence of high-dose D-allethrin, and a correlation was found precisely between the enzyme activity reflected by the final frequency shift after the hydrolyzation and the concentration of D-allethrin, with a significant frequency response observed even at a 1.5×10^{-5} volume ratio of D-allethrin to distilled water. The present work may have presented a novel method feasible for study on the mechanism of excitability of spiders under low-dose D-allethrin pesticide and quick detection of proteinase activity.

Keywords: *Pardosa pseudoannulata*, D-allethrin, proteinase activity, ultraviolet spectroscopy, piezoelectric bulk acoustic wave impedance method.

Spiders are the important predatory natural enemies of rice insect pests and play an important role in the ecological regulation and management of insect pests in rice fields[1]. In 2002, we found that low-dose chemical pesticides may strengthen ability of spiders for controlling insect pests and hypothesized that low-dose chemical pesticides could strengthen insect-control ability of spider[2]. But until now, the hypothesis has not yet been verified.

The proteinase exists naturally in the midgut of spiders and it has very strong specificity to substrates. Its activities have a direct effect on the predatory ability of spiders. So it is necessary to undertake a further study on the effect of low-dose chemical pesticides on the proteinase activity of spider. Various techniques have been used to study enzyme activity detection, e. g. UV-Vis spectrophotometry, chemiluminescence and agarose-gel electrophoresis. However, these traditional methods have some shortcomings, such as requiring harmful or radioactive chemicals, complicated operation skills, limitations for determining various types of damages besides single- and double-strand breaks, and difficulty in providing multidimensional information *in situ*. In our present work bulk acoustic wave (BAW) impedance analysis, a new technique providing multidimensional information *in situ*, was applied to investigate the proteinase-catalyzed hydrolyzation of casein and the effect of pesticide D-allethrin on the proteinase activity in the midgut of the spider *Pardosa pseudoannulata*. The method has wide applications in the interaction process of protein[3] and enzymatic kinetics[4]. However, this paper is the first report about proteinase-catalyzed hydrolyzation of specific substrate and the effect of pesticide D-allethrin on the proteinase activity.

1 Theoretical foundation of the piezoelectric BAW impedance method

Since the early 1980s, the piezoelectric quartz crystal (PQC) has been found stable in the liquid phase and widely applied to *in situ* monitoring of the chemical / biological processes near the electrode sur-

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face[5,6]. PQC sensor mainly uses its bulk acoustic wave (BAW) to spread for a micrometres distance in the liquid phase and it is sensitive to the mass change of the electrode surface. So PQC sensor is also called quartz crystal microbalance (QCM) or bulk acoustic wave (BAW) sensor. With the advantages such as high sensitivity, simplicity of use, and the capability of providing multidimensional information on physical and/or chemical properties of the investigated system, applications of piezoelectric BAW impedance analysis technique have been developed recently for many areas in life science, including bacteria detection[7], protein chemistry[8], nucleic acid chemistry[9], immunization dynamics[3,10], enzyme dynamics[4], and so on. In this work, we developed a novel method for the volant detection of enzyme activity. Of the AT-cut BAW quantitative sensor theory in the liquid phase, the following two equations are very important: Sauerbrey equation (1) describes a linear frequency-mass relationship during loading and removal of a rigid, thin and homogeneous film on the PQC electrode[11]; Martin equation (2) describes a net Newtonian-liquid loading effect (net viscous effect)[12].

$$\Delta f_0 = \frac{2f_{0g}^2}{(\rho_Q \mu_Q)^{1/2}} \frac{\Delta m}{A} = -2.264\times 10^{-6} f_{0g}^2 \frac{\Delta m}{A} \quad (1)$$

where f_{0g} is the resonant frequency unperturbed PQC and Δf_0 is the resonant frequency change upon transferring the PQC from a vacuum to a solution; ρ_Q and $\bar{u}_Q$ are the density (2.651 g/cm^3) and shear modulus (2.947×10^{10} N/m^2), respectively; A and Δm are the piezoelectrically active area of electrode and mass change of the electrode surface, respectively.

$$\Delta R_{1L} = -4\pi L_Q \Delta f_{0L} \sqrt{f\mu_Q}/\sqrt{f_{0g}\bar{c}_{66}} \approx -4\pi L_Q f_{0L} \quad (2)$$

where Δf_{0L} and ΔR_{1L} are the resonant frequency (f_0) changes and motional resistance (R_1), respectively; f is the center frequency of quartz crystal; $\bar{c}_{66}$ is the piezoelectrically stiffened elastic constant (2.957×10^{10} N/m^2) for AT-cut thickness-shear-mode (TSM) PQC; L_Q is the motional inductance of the quartz crystal in the air.

According to Eq. (2), the characteristic slope value of $\Delta f_{0L}/\Delta R_{1L}$ for a net density/viscosity effect on the 9 MHz PQC resonance is approximately -10 Hz·Ω^{-1}, namely, for a change of 1 Ω in R_1 due to the solution density-viscosity effect, the f_0 change would be about 10 Hz in theory. However, in practice, the absolute value of this ratio would be bigger because of the mass-loading effect on the f_0 response. According to previous reports on the responses of the PQC sensor to the density-viscosity change, if the experimental value of the ratio was very close to the theoretical calculation over this reaction process, it might be that the changes of PQC responses we observed mainly came from the variation in density-viscosity of the tested solution caused by the proteinase-catalyzed hydrolyzation of casein. Otherwise, the changes of PQC responses are chiefly due to the variation in the mass-loading effect of the gold electrodes surface immersed in the test solution. Obviously, the larger the absolute value of $\Delta f_{0L}/\Delta R_{1L}$, the weaker the viscous effect and the stronger the mass effect.

2 Materials and methods

2.1 Apparatus

The schematic of the experimental setup is illustrated in Fig. 1. A 9 MHz AT-cut PQC (12.5 mm in diameter) was used, which has gold electrodes on both sides: one side paced in air and the other side immersed in the test solution. The conductance (G) and susceptance (B) data of the BAW/PQC sensor were recorded synchronously on a Hewlett-Packard (HP) 4395A network/spectrum/impedance analyzer interfaced to an IBM-compatible personal computer. Based on the simultaneous fitting of both G and B data to the BVD equivalent circuit via an HP-IB interface card for windows 3.1/NT/95, admittance (Y) data were acquired in real time by a program written in Visual Basic 5.0 that controlling the HP 4395a analyzer. Other equivalent circuit parameters were obtained with the same program.

The test solutions were held in a 5 mL detection cell and stirred at a constant rate with a magnetic stirrer. All experiments were carried out at 28±0.5 ℃.

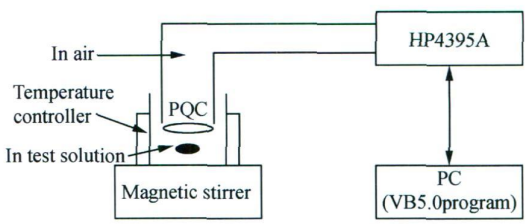

Fig. 1. The schematic diagram of piezoelectric quartz crystal impedance (PQCI).

2.2 Materials

The adult female spiders, *Pardosa pseudoannulata*, were collected from bio-controlling rice fields and then raised in the laboratory.

The low-dose pesticides was prepared by diluting 93% D-allethrin pesticide with distilled water to a concentration of 0.03%. Three representative pesticide concentrations (volume ratio of pesticide to water: 1∶20, 2∶20, 6∶20) were selected based on the observation of the predatory functional response of *P. pseudoannulata to Nilaparvata lugens*, the latter is a main insect pest in rice fields.

2.3 Methods

2.3.1 Extraction of proteinase sources Two or three female adults of *P. pseudoannulata* after 48 hours hunger were dissected under a stereo microscope in a cold water bath. The midgut was taken out and homogenated in 2 mL phosphate buffer solution at pH 8.0. The homogenate was centrifuged at 4000 g for 15 min at 0—4 ℃. The supernatant was taken as proteinase sources.

2.3.2 Measurement of the protein concentration The method of Bradford[13] was used to determine the protein concentration.

2.3.3 BAW impedance analysis of proteinase activity A buffer solution of 1 mmol/L PBS (pH7.5) containing 0.1 mL (0.5%) casein was brought into the test cell, then the initial steady resonant frequency (f_{01}), motional resistance (R_{11}), motional inductance (L_{11}) and static capacitance (C_{01}) were recorded as the reference. Subsequently, proteinase (0.2 mL) followed by different concentrations of pesticide (0.1 μL) were injected into the buffer. During the reaction, the values of resonant frequency (f_0), motional resistance (R_1), static capacitance (C_0) and motional inductance (L_1) were measured synchronously after the addition of pesticide. The changes of equivalent circuit parameters ($\Delta f_0 = f_0 - f_{01}$, $\Delta R = R_1 - R_{11}$, $\Delta L_1 = L_1 - L_{11}$ and $\Delta C_0 = C_0 - C_{01}$) were plotted against the reaction time. The response curves of the BAW sensor in the process were obtained. After that, these parameters reached platforms again and the terminal stable values were remeasured as f_{02}, R_{12}, and L_{12}, respectively. The end-point changes were recorded as $\Delta f_m (= f_{02} - f_{01})$, $\Delta R_m (= R_{12} - R_{11})$, $\Delta L_m (= L_{12} - L_{11})$ and $\Delta C_m (= C_{02} - C_{01})$, respectively. By measuring the change of frequency, the extent of the proteinase-catalyzed hydrolyzation of casein was monitored *in situ*. The initial velocity $v (\Delta f/t)$ of proteinase-catalyzed hydrolyzation was obtained by the curve $\Delta f - t$.

2.3.4 Ultraviolet spectroscopy of proteinase activity The 0.5 mL (0.5%) casein containing proteinase (0.2 mL) plus 0.015 μL low-dose pesticide were added into the test cell and mixed. After 15 min reaction in a 37 ℃ water bath, 1.3 mL 20% Trichloro acetic acid (TCA) was injected into the test cell to end the reaction and the reaction was equilibrated for 2 min. Subsequently, the reactions mixture was centrifuged for 10 min at 4000 *g* at 0—4 ℃. The 0.8 mL supernatant were taken out after the addition of 1.6 mL Na_2CO_3 plus 0.2 mL Folin-phenol. The absorption spectra of the DNA at 680 nm were recorded. The measurement was repeated three times.

3 Results and discussions

3.1 Proteinase-catalyzed hydrolyzation detected by BAW impedance analysis

Curves in Fig. 2 show the changes in the equivalent circuit parameters of PQC (Δf_0, ΔR_1, ΔC_1, ΔL_1) against the reaction time during the proteinase-catalyzed hydrolyzation process without D-allethrin pesticide. The addition of proteinase resulted in an evident increase in Δf_0 first which then gradually reached to a relatively steady value (i.e., Δf_m). Meanwhile, ΔR_1 and ΔL_1 decreased inversely, and ΔC_1 increased slightly. As shown in Fig.3, simultaneous responses of Δf_0 and ΔR_1 of the BAW sensor are similar to the former in the background system with D-allethrin (the volume ratio of pesticide to water was 1∶20). But the end-point frequency increases evidently. This observation shows that the stranger of the enzyme activity, the more casin being hydrolysed. The curve changes of ΔC_1 and ΔL_1 are relatively steady. To understand the response mechanism of BAW impedance analysis, the value of $\Delta f_0/\Delta R_1$ was calculated, which were 11.2 Hz·Ω^{-1} and 12.5 Hz·Ω^{-1} in the background system with D-allethrin and without D-allethrin, respectively. The result is very close to the characteristic slope value of $\Delta f_{0L}/\Delta R_{1L}$. According to previous reports[14] on the responses of the PQC sensor to the density-viscosity change, it is demonstrated that the changes of PQC responses in

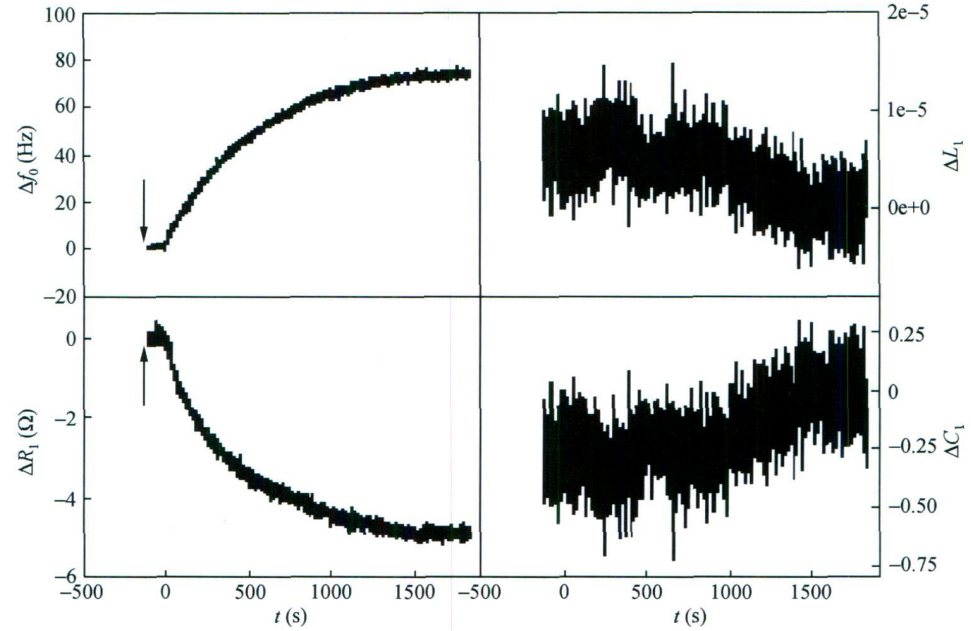

Fig. 2. Simultaneous responses of Δf_0, ΔR_1, ΔL_1 and ΔC_0 of the BAW sensor following the addition of proteinase (0.2 mL) into the buffer solution containing 0.1 mL (0.5%) casein. The arrows show the addition of proteinase (0.2 mL) into the 5-mL detection cell.

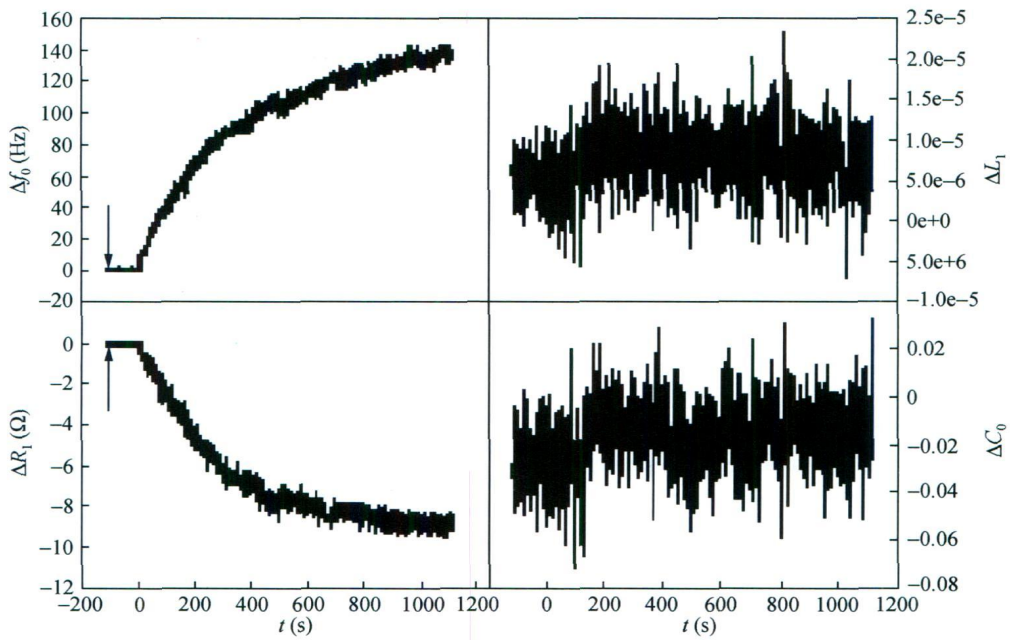

Fig. 3. Simultaneous responses of Δf_0, ΔR_1, ΔL_1 and ΔC_0 of the BAW sensor following the addition of proteinase (0.2 mL) into the buffer solution containing 0.1 mL (0.5%) casein plus 0.1 mL D-allethrin (1∶20). The arrows show the addition of proteinase (0.2 mL) into the 5-ml detection cell.

the present work are chiefly due to the variation in density-viscosity of the tested solution in the proteinase-catalyzed hydrolyzation process.

3.2 The effect of D-allethrin concentrations on hydrolyzation

Fig. 4 shows simultaneous responses of Δf_0 with the addition of D-allethrin on the same casein and proteinase.

When the volume ratio of pesticide to water was 1∶20 and 2∶20, the Δf_0 vs time response curve of PQC moved to the left to different extent. The range of increasing was greater in comparison to that of no

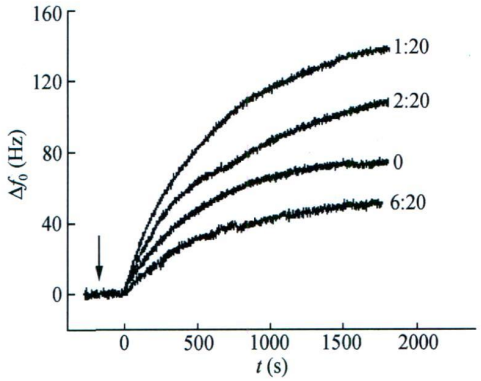

Fig. 4. Simultaneous responses of Δf_0 the BAW sensor following the addition of proteinase (0.2 mL) in buffer solution containing 0.1 mL (0.5%) casein plus D-allethrin at different concentrations. The arrow shows the addition of proteinase (0.2 mL) into the detection cell.

pesticide. The slope rate of curve, the range of increasing and the activation degree of enzyme were the greatest at the ratio of 1∶20. This showed that casein had the most complete hydrolysis ability and the density of the solution had the biggest changes on the condition of 1∶20. Whereas under the condition of high-dose D-allethrin (the volume ratio of pesticide to water was 6∶20), Δf_0 vs time response curve moved to the right to different extent. The slope and range of curve obviously diminished. The ascending speed of frequency slowed and enzyme activity was significantly inhibited. The mechanism may be that the conformations of proteinase might change in the presence of low-dose D-allethrin and its active center might tend to combine with the substrates. This made protein-enzyme compound form easily and enzyme activity increased; but the conformations of proteinase became compact in the presence of high-dose D-allethrin (such as 6∶20) and its active center was more difficult to combine with the substrates. This decreased enzyme activity, and the enzymatic action slowed. The exact mechanism of activation or inhibition effect of pesticide on proteinase activity in spider's midgut is needed to be studied further. Fig. 5 shows the relationship between the change of the end-point frequency and the concentration of D-allethrin in the system containing casein and proteinase in spider's midgut.

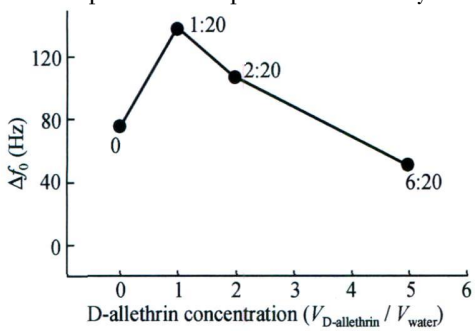

Fig. 5. Relationship between end-point frequency change (Δf_m) and D-allethrin concentration in the system containing casein and proteinase in spider's midgut.

3.3 Ultraviolet spectroscopy analysis

Table 1 shows the effect of different low-dose D-allethrin on midgut proteinase activity of *P. pseudoannulata*.

From Table 1, it can be known that when the ratio of pesticide to water was 1∶20, 2∶20 and 6∶20, the enzyme activity ratio of pesticide to no-pesticide was 1.45, 1.12 and 0.61, respectively. It was statistically significant or very significant showed by *t*-test. The proteinase activity increased in the presence of suitable low-dose D-allethrin, but was inhibited in the presence of high-dose D-allethrin, implying a comformational change of the proteinase in the presence of low-dose D-allethrin makes it easy to combine with the substrates. It is also favorable for degradation of the substrates. With the increasing of pesticide concentration, the proteinase activity decreased gradually. The results were the same to those of BAW monitored.

Table 1. Effect on proteinase activity in the midgut of *P. pseudoannulata* under different low-dose D-allethrin

Pesticide∶water	Midgut proteinase activity	Ratio
0	0.0326±0.0015	1
1∶20	0.0473±0.0029**	1.45
2∶20	0.0365±0.0027*	1.12
6∶20	0.0198±0.0007**	0.61

Note: By *t*-test, * stands for significantly different at $P < 0.05$, ** stands for significantly different at $P < 0.01$.

4 Conclusion

The results of the present investigation demonstrate that the BAW/impedance method has been successfully applied to analyze and assay the proteinase-catalyzed hydrolyzation of casein and to study the effect of pesticide D-allethrin on the proteinase activity in the midgut of the spider *Pardosa pseudoannulata*. With the coexistence of pesticide D-allethrin and casin, the hydrolyzation was significantly inhibited at higher concentrations but significantly increased at lower concentrations, and this was in good agreement with the results obtained by other methods under similar conditions. Compared with other conventional methods, the BAW/impedance method has the ad-

vantages of high sensitivity, simplicity in operation, convenience in automation and requiring not harmful or radioactive chemicals. Meanwhile, an obvious correlation was found between the enzyme activity reflected by the final frequency shift after the hydrolyzation with D-allethrin, and a significant frequency response was observed even at a 1.5×10^{-5} volume ratio of D-allethrin to distilled water. Additionally, the proposed method can provide the real-time multidimensional information and is capable of quantitatively reflecting the relationship of the sensor responses and the sensor-solution interracial process, which makes it possible to use for on-line analysis of the investigated system over the whole reaction process. Moreover, if used in combination with a flow injection system or microdialysis technique, etc., the BAW/impedance device will offer great potential for developing a powerful tool for *in situ* and *in vivo* monitoring of the biological processes in wider areas such as dynamic analytical biochemistry.

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