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Short communication

Potentiometric determination of trypsin using a polymeric membrane polycation-sensitive electrode based on current-controlled reagent delivery

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ABSTRACT

A potentiometric biosensor for the determination of trypsin is described based on current-controlled reagent delivery. A polymeric membrane protamine-sensitive electrode with dinonylnaphthalene sulfonate as cation exchanger is used for *in situ* generation of protamine. Diffusion of protamine across the polymeric membrane can be controlled precisely by applying an external current. The hydrolysis catalyzed with trypsin in sample solution decreases the concentration of free protamine released at the sample–membrane interface and facilitates the stripping of protamine out of the membrane surface via the ion-exchange process with sodium ions from the sample solution, thus decreasing the membrane potential, by which the protease can be sensed potentiometrically. The influences of anodic current amplitude, current pulse duration and protamine concentration in the inner filling solution on the membrane potential response have been studied. Under optimum conditions, the proposed protamine-sensitive electrode is useful for continuous and reversible detection of trypsin over the concentration range of 0.5–5 U mL^{-1} with a detection limit of 0.3 U mL^{-1}. The proposed detection strategy provides a rapid and reagentless way for the detection of protease activities and offers great potential in the homogeneous immunoassays using proteases as labels.

1. Introduction

Proteases, a unique class of enzymes, play an important role in many physiological and pathological processes including protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, and tumor growth and metastasis [1–5]. Therefore, the determination of protease activities is of great importance from the physiological and pathological points of view. Nowadays, various chemical sensors have been prepared using proteases for cleaving peptide chains followed by appropriate signal transductions including spectrophotometry [6], colorimetry [7], fluorimetry [8], radiometry [9], holography [10], chromatography [11], amperometry [12], alternating current impedance [13], conductometry [14], and potentiometry [15]. Among these sensors, the potentiometric polyion sensor has appeared very promising for monitoring protease activities [16–20]. The potentiometric response of such a polyion sensor configuration is governed by a non-equilibrium steady-state extraction of the polyion into the organic membrane phase of the electrode via formation of cooperative ion pairs with lipophilic ion exchangers in the membrane phase [21]. The protease activity can be detected by measuring the concentration of protamine as it is digested by the enzyme into smaller fragments to which the potentiometric polyion sensor is less sensitive. Compared to other techniques, the polyion sensitive membrane electrode has great potential for on-site monitoring due to its favorable portability, short analysis time, inexpensive equipment, and the ability to detect protease activities in samples as complex as plasma and whole blood. However, manual addition of the substrates to the sample solution is always required before analysis. A reagentless detection mode is highly desired to meet the growing demands for *in situ* monitoring of protease activities.

In this communication, a potentiometric biosensor for the determination of trypsin is described. It is based on trypsin-catalyzed degradation of protamine which is released from the inner solution of a protamine-conditioned polycation-sensitive electrode. Diffusion of protamine across the polymeric membrane can be controlled precisely by applying an external current. The hydrolysis catalyzed with trypsin in the sample solution decreases the concentration of free protamine released at the sample–membrane interface. This would facilitate the stripping of protamine out of the membrane surface via the ion-exchange process with sodium ions from the sample solution, thus decreasing the membrane potential, by which trypsin can be sensed potentiometrically. Compared with previous protease

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sensing strategies, our potentiometric detection scheme offers a rapid and reagentless way for real-time detection of protease activities.

2. Experimental

2.1. Reagents

Protamine sulfate salt from herring, trypsin from bovine pancreas (13,000 U/mg), tetrahydrofuran (THF) and tris(hydroxymethyl)-aminomethane (Tris) were obtained from Sigma (St. Louis, MO). 2-nitrophenyl octyl ether (*o*-NPOE), tetradodecylammonium tetrakis(4-chlorophenyl)borate (ETH 500), high molecular weight poly(vinyl chloride) (PVC), and dinonylnaphthalene sulfonate (DNNS) as 50% solution in heptane were purchased from Fluka AG (Buchs, Switzerland). Aqueous solutions were prepared with freshly deionized water (18.2 MΩ specific resistance) obtained with a Pall Cascada laboratory water system.

2.2. Membrane preparation

The protamine-conditioned polycation-sensitive membrane contained 29 wt.% PVC, 58 wt.% *o*-NPOE, 3 wt.% lipophilic cation exchanger DNNS and 10 wt.% lipophilic salt ETH 500. The membrane was obtained by casting a solution of ~200 mg of the membrane components dissolved in 3.0 mL tetrahydrofuran (THF) into a glass ring of 3.6 cm diameter fixed on a glass plate and letting the solvent evaporate overnight. For each ion selective electrode (ISE), a disk of 7 mm diameter was punched from the membrane and glued to a plasticized PVC tube (i.d. 6 mm, o.d. 9 mm) with THF/PVC slurry. A solution of 0.05 mg mL^{-1} protamine containing 120 mM NaCl and 50 mM Tris–HCl buffer (pH 7.4) was used as the inner filling solution of the protamine-conditioned polycation-sensitive electrode [22]. Before measurements, the electrode was conditioned for 12 h in a solution identical to the inner filling solution at 25 ± 1 °C. When not in use, the sensor was stored in the conditioning solution at 4 °C.

2.3. Experimental setup

All the measurements were carried out in 50 mM pH 7.4 Tris–HCl buffer solution containing 120 mM NaCl at 25 ± 1 °C using a conventional three-electrode system with an ISE electrode, a platinum auxiliary electrode, and an Ag /AgCl (3 M KCl) reference electrode. Experiments were performed on the CHI 760C electrochemical workstation (shanghai, China) and were controlled by a macro-command, which executes a series of commands in the specified order (Fig. 1A). The procedures for switching between the galvanostatic and potentiostatic steps were designed as described before [22,23]. The open-circuit potential of the electrode was first recorded for 1 s (pulse 1). Then, an anodic current of 1 μA with a duration of 3 s was applied for protamine release (pulse 2), and the system was interrogated at zero-current for 60 s to avoid any undesired IR drop [20] (pulse 3). Finally, potentiostatic voltage control at the open-circuit potential of the electrode in the absence of analyte was used to refresh the membrane with a recovery time of 120 s (pulse 4). To eliminate the disturbance of the protamine diffusion through the polymeric membrane, all the measurements were done without sample stirring. During the galvanostatic pulse, the potential was monitored as a function of time and the current-dependent potential difference (ΔE) at 3 s between the potentials measured before and after addition of trypsin was used for quantification of enzyme activities. For better reproducibility, the first series of pulses 1–4 for the fresh membrane was ignored and the potential readings during the sequent pulses were used for the measurements.

3. Results and discussion

3.1. Analytical principle

Recently, we developed a promising potentiometric biosensing system using pulsed-current-driven reagent delivery for controlled-release of substrate ions at the sample–membrane interface, which

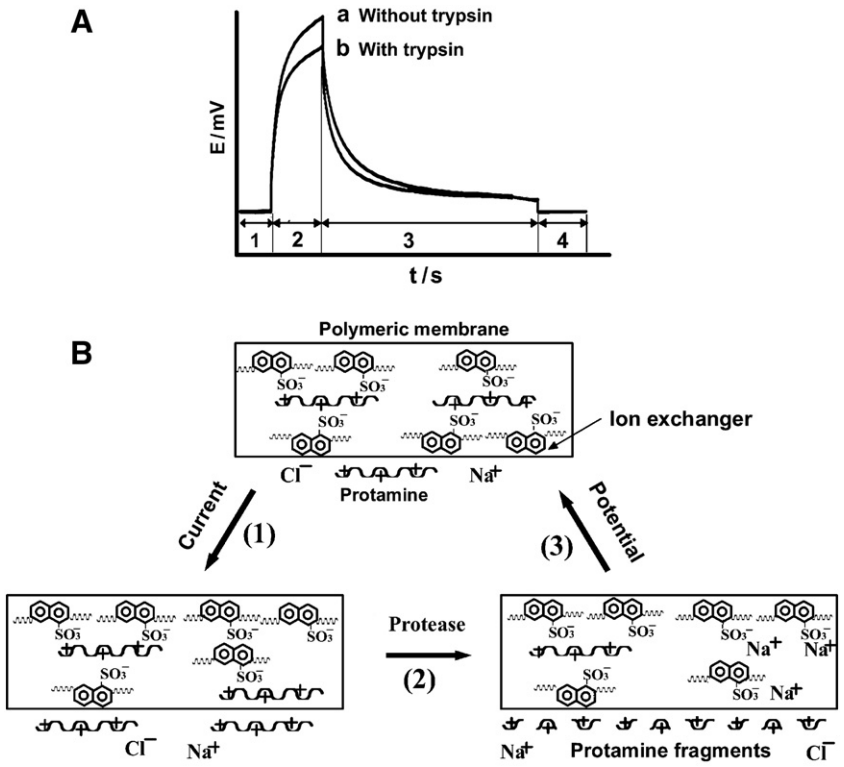

Fig. 1. (A) Potentiometric responses of the polycation-sensitive membrane electrode with current-controlled release of protamine in buffer solutions without trypsin (a) and with trypsin (b). (B) Scheme of the proposed potentiometric sensing principle for protease detection.

enables rapid, reproducible and continuous sensing of enzyme activities and screening of their inhibitors and activators [23]. In our previous research, the enzyme substrates are restricted to singly charged reagents. Herein, protamine, a polypeptide rich in arginine residues which has a charge of about +20 [24], is used as a substrate for detection of protease. Using trypsin as a model of protease, it has been found that the current-controlled release of protamine enables sensing of trypsin in a rapid and reproducible way.

As illustrated in Fig. 1B, the proposed potentiometric detection of trypsin involves three steps: (1) an applied current drives the ion fluxes of protamine through the polymeric membrane; (2) the free protamine released at the sample–membrane interface is degraded by trypsin; and (3) a uniform protamine concentration profile in the membrane phase can be quickly established by applying the external voltage for membrane recovery. Fig. 1A shows that the current-dependent potential decreases with increasing the concentration of trypsin in the sample solution, which is due to the decrease of the concentration of free protamine released at the sample–membrane interface via the degradation by trypsin. As described before [18,22], the consumption of protamine would facilitate the stripping of protamine out of the membrane surface via the ion-exchange process with sodium ions from the sample solution, which can dramatically decrease the potential values. Fig. 1A also indicates that a potential decrease by the hydrolysis can be observed at both current-dependent potential (pulse 2) and zero current potential (pulse 3). As compared to the response with the zero current potential, a much larger potential increase can be obtained with the current-dependent potential. This is probably due to the relatively high catalysis activities of trypsin, thus causing rapid potential responses even during the applied current pulse.

3.2. Optimization for measuring trypsin with the protamine-conditioned polycation-sensitive electrode

Since the polycation-sensitive membrane electrode shows the sigmoidal relationship between the potential measured and the protamine concentration [25], optimization was made for the protamine release. The influence of the applied current on the potential difference was tested with 0.1 μA, 0.5 μA, 1 μA and 2 μA for measuring 2.5 U mL^{-1} trypsin (Fig. 2A). Experiments showed that the maximum value could be obtained when the anodic current of 1 μA was applied. The influence of current pulse duration on the potential difference was tested with trypsin of 2.5 U mL^{-1} (Fig. 2B). It was found that the potential difference of the electrode for trypsin increased rapidly with increase in the current pulse duration until 3 s, and then the potential difference was nearly constant. This is probably due to the fact that a constant release of protamine could be available after 3 s. Therefore, the current pulse duration of 3 s was chosen. Since the amount of protamine released at the sample–membrane interface is dependent on the concentration of protamine in the inner solution of the electrode, one can tune the measuring range and improve the detection limit of the sensor by selecting the inner filling solution with a suitable protamine concentration [26]. The influence of protamine concentration in the inner solution on the measured potential difference was tested for trypsin in the range of 2.5–7.5 U mL^{-1}. As shown in Fig. 2C, with 0.01 mg mL^{-1} or 0.1 mg mL^{-1} protamine, the potential difference is rather small, while with 0.05 mg mL^{-1} protamine, a large potential difference can be obtained (*ca.* 20 mV for 5 U mL^{-1} trypsin). Thus, 0.05 mg mL^{-1} protamine in 50 mM pH 7.4 Tris buffer was used as the inner filling solution.

3.3. Performance of the protamine-conditioned polycation-sensitive electrode

The calibration curve of the proposed trypsin sensor was derived from the differential potentiometric response curves obtained at the protamine-conditioned polymeric membrane polycation-selective

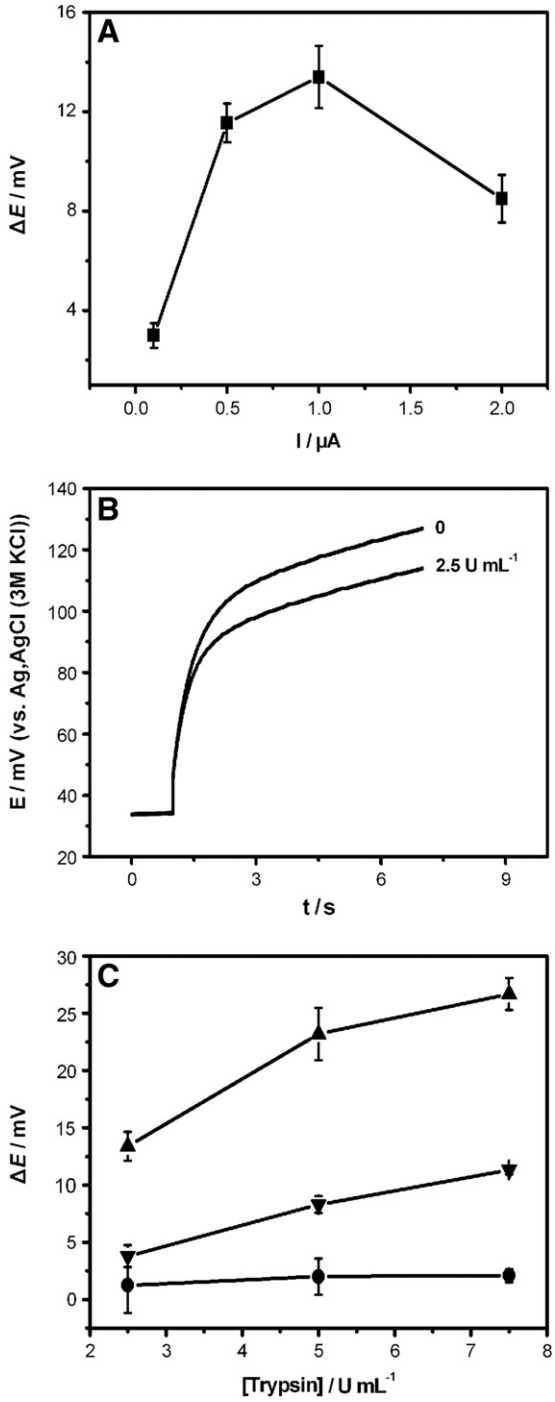

Fig. 2. Potential responses of the protamine-sensitive electrode to trypsin (2.5 U mL^{-1}) with (A) different anodic current amplitudes and (B) different current pulse durations. (C) Potential responses of the protamine-sensitive membrane electrodes to trypsin with (●) 0.01, (▲) 0.05, and (▼) 0.1 mg mL^{-1} protamine in the inner filling solutions. The membranes were composed of 29 wt.% PVC, 58 wt.% *o*-NPOE, 3 wt.% lipophilic cation exchanger DNNS and 10 wt.% lipophilic salt ETH 500. Error bars represent one standard deviation for three measurements.

electrode with a current pulse duration of 3 s and an anodic current amplitude of 1 μA (Fig. 3(A)). The potential difference is proportional to the concentration of trypsin in the range of 0.5–5 U mL^{-1} ($\Delta E = 4.49\,C + 1.54$, $r = 0.9973$, ΔE in mV, C in U mL^{-1}). For 5 U mL^{-1} trypsin, the relative standard deviation (RSD %) is 8.0% (n = 6). The detection limit is given by the equation $C_L = 3s_{bl}/S$, where s_{bl} is the standard deviation of the blank measurements and S is the sensitivity of the calibration graph. The detection limit

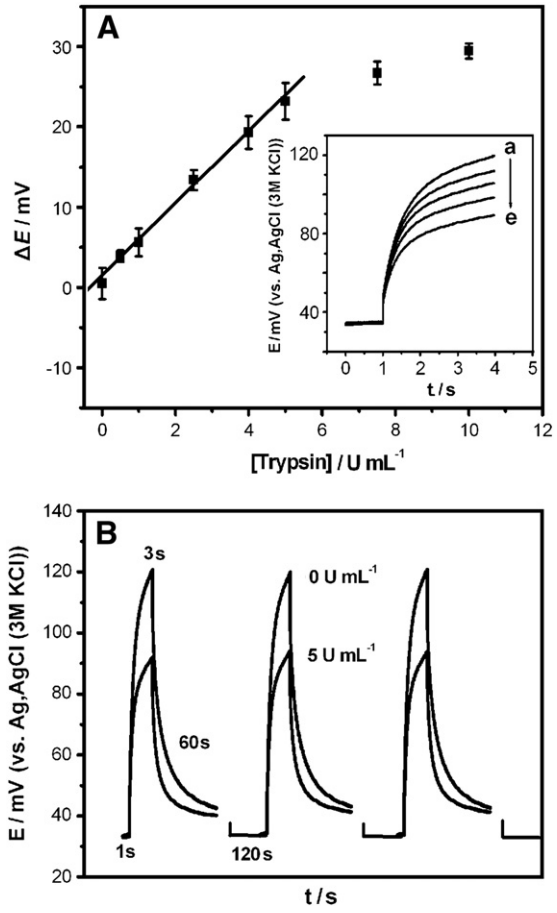

Fig. 3. (A) Calibration curve of the protamine-sensitive membrane electrode for detection of trypsin. The inset shows potentiometric responses of the protamine-sensitive membrane electrode in 50 mM pH 7.4 Tris buffer containing 120 mM NaCl upon additions of trypsin of (a) 0, (b) 1, (c) 2.5, (d) 4, (e) 10 U mL^{-1}. Error bars represent one standard deviation for three measurements. (B) Recycle response profiles for the blank and a trypsin solution. Other conditions are the same as in Fig. 2.

of trypsin was calculated to be 0.3 U mL^{-1}, which is comparable to or better than those obtained by other potentiometric sensors [16,18,20]. Experiments showed that no loss of potential response was observed after 100 measurements with the same internal solution and the same membrane.

Reversibility of the protamine-sensitive membrane electrode was evaluated by measuring the alternating responses to the buffer and the trypsin solutions. The results are shown in Fig. 3(B). It can be seen that the potential response is reversible from pulse to pulse and the signal changes are fully repeatable. For 5 U mL^{-1} trypsin, the time to achieve 98% of the full-scale response is very fast (3 s), and the recovery to 98% of the initial value takes ca. 3 min. This is probably due to the fact that the release of substrate ions at the membrane–sample interface can be quickly renewed by applying the external voltage. It should be noted that trypsin can also be detected by using the protamine-conditioned polymeric membrane under zero-current conditions. However, it has been found that the spontaneous release of protamine through the ISE membrane would induce a rather poor reversibility, probably due to the difficulty in the renewal of the protamine concentration profile in the membrane phase.

4. Conclusions

In summary, a polymeric membrane polycation-sensitive electrode for trypsin has been developed based on pulsed-current-driven reagent delivery. The proposed method offers a rapid, reversible and reagentless way for protease detection. Such a sensing scheme can provide an excellent platform for sensing of protease activities and has promising potential in the homogeneous immunoassays using proteases as labels [27,28].

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