Microchim Acta (2015) 182:43–49
DOI 10.1007/s00604-014-1295-5

ORIGINAL PAPER

Electrochemical determination of trypsin using a heptapeptide substrate self-assembled on a gold electrode

Manman Dong · Honglan Qi · Shengen Ding · Min Li

Received: 26 March 2014 / Accepted: 14 May 2014 / Published online: 5 June 2014

Abstract A simple and sensitive electrochemical method was developed for the determination of trypsin by employing a specific heptapeptide (CRRRRRR) as a substrate. The positively charged heptapeptide substrate is self-assembled on the surface of a gold electrode through the thiol group of the cysteine (C) at the end of the peptide, which prevents the electrochemical probe [the ruthenium(III) hexammine complex] to access the electrode. The substrate peptide is hydrolyzed by trypsin, and this causes the fragments to leave the electrode and, consequently, the electrochemical signal to increase. The results show that the increase in the square wave voltammetric current of the ruthenium probe is linearly related to the activity of trypsin in the range from 0.0047 to 0.052 U·mL^{-1} with a detection limit of 0.0012 U·mL^{-1}. This work demonstrates that the enzymatic cleavage of the substrate can be directly converted to an electrical signal to provide a simple and sensitive method for the determination of trypsin.

Keywords Electrochemistry · Peptide · Trypsin · Cleavage

Introduction

Trypsin is a serine protease produced by the pancreatic acinar cells and it can cleave peptide bonds on the C-terminal side of lysine or arginine amino acid residues. The initial proenzyme form of trypsinogen can self-cleave to generate the active form of trypsin, which then induces the transformation of other pancreatic proenzymes into the active forms [1]. Therefore, trypsin plays a critical role in the control of pancreatic exocrine function and trypsin serves as a reliable and specific biomarker for pancreatic function and its pathological changes [2]. To develop simple, selective and sensitive methods for the determination of trypsin has attracted much attention in recent year. Various methods for the determination of trypsin have been developed in the past few years, such as radioimmunoassay [3], enzyme-linked immunosorbent assay [4], colorimetric [5, 6] fluorescent [7, 8] and electrochemical methods [9–11]. Among them, electrochemical methods have received much attention because they provide simple, inexpensive and accurate platform for the measurement of the target analyte [12]. Most of electrochemical methods for the determination of trypsin are mainly focused on the improvement of sensitivity by employing various amplification strategies. For example, Ionescu et al. reported an amperometric biosensor for the detection of trypsin on basis of enzyme amplification [10]. Although the sensitivity is improved, these methods still have respective disadvantages, such as complicated labelling and analytical procedures; therefore, it is still required to develop simple, sensitive methods for the determination of trypsin, especially in the point-of-care applications.

Here, a simple and sensitive electrochemical method for the determination of trypsin was developed by employing a specific heptapeptide (CRRRRRR) as substrate. The substrate peptide (CRRRRRR) was designed according to refs [13, 14], in which contains six arginines with six cationic charges (p*I*≈ 10.8) and a thiol group of the cysteine (C) at the end of the specific peptide to facilitate self-assembly onto the surface of gold electrode [15]. Fig. 1 presents the schematic diagram of the electrochemical method for the determination of trypsin. The positively charged heptapeptide substrate is self-assembled on the surface of a gold electrode through the thiol group of the cysteine at the end of the peptide and blocks the penetration of the positive electroactive indicator, hexammine ruthenium (III) chloride ($[Ru(NH_3)_6]^{3+}$). When trypsin is present, it specifically hydrolyzed the specific peptide, led

M. Dong · H. Qi (✉) · S. Ding · M. Li
Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710062, People's Republic of China
e-mail: honglanqi@snnu.edu.cn

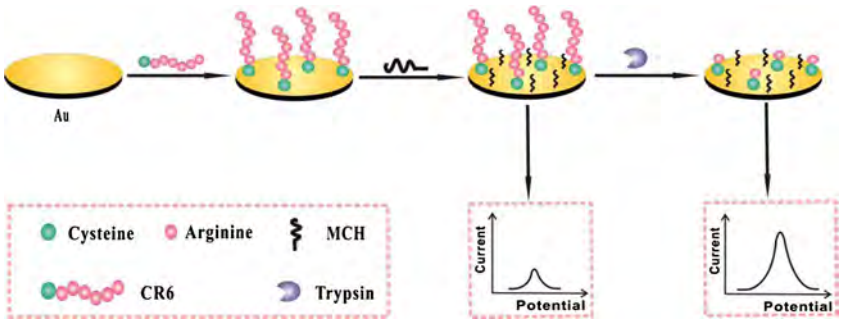

Fig. 1 Schematic diagram of the electrochemical method for the determination of trypsin

the peptide to leave the electrode, and resulting in the increase of the electrochemical signal obtained from the resulted electrode in 10 mM Tris-HCl (pH 7.4) containing $[Ru(NH_3)_6]^{3+}$. The design and characteristics of the electrochemical method are discussed and the analytical performance for the determination of trypsin is presented.

Experimental

Reagent

The peptide (CRRRRRR, CR6) was designed according refs 13 and 14, and chemically synthesized by ChinaPeptides Co. LTD (www.chinapeptides.com, China). Trypsin was obtained from Xi'an Wolsen Bio-technology Co., Ltd. (www.wolsen.com, China). 6-Mercapto-1-hexanol (MCH), thrombin, glutathione reductase (GR) and acetylcholine esterase (AchE) were obtained from Sigma-Aldrich (www.sigmaaldrich.com, USA). Hexammine ruthenium (III) chloride ($[Ru(NH_3)_6]^{3+}$) was purchased from Acros Organics Corporation (www.inno-chem.com.cn/acros, Belgium). 0.1 M phosphate buffer saline consisted of 0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4 and 0.1 M KCl (PBS, pH 7.4). 10 mM phosphate buffer (PB, pH 7.4) containing 10 mM NaH_2PO_4 and 10 mM Na_2HPO_4 was used as washing solution. 10 mM Tris-HCl (pH 7.4) was used as detection buffer solution. All chemical reagents were of analytical grade and used as received, and Millipore Milli-Q water (18.2 MΩ·cm) was used.

Apparatus

All electrochemical experiments including cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and square-wave voltammetry (SWV) were carried out using a CHI 660 electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd. China, http://www.chinstr.com). A conventional three-electrode system, which consisted of a gold electrode (2.0 mm diameter) or a peptide-modified electrode as the working electrode, a platinum wire as the counter electrode, and a Ag/AgCl (saturated KCl) as the reference electrode was used. All potentials in this work were referred to the reference electrode. Atomic force micrograph (AFM) images were obtained with a CSPM5500 Scanning Probe Microscope (Being Nano-Instruments, ltd. China, http://www.gongchang.com).

Preparation of peptide modified electrode

Prior to the experiment, the gold electrode was polished with 0.3 μm alumina slurry and then ultra-sonicated in water for 5 min, and then cleaned electrochemically by a linear scanning potential between 0 and +1.5 V in 0.10 M H_2SO_4 until a stable cyclic voltammogram was obtained [16].

The cleaned gold electrode was dipped into 100 μL of 10 $\mu g.mL^{-1}$ peptide solution for 40 min and washed thoroughly with 10 mM PB (pH 7.4) to remove unbound peptide. After that, the resulting electrode was immersed into 1 mM MCH solution for 30 min and washed with 10 mM PB (pH 7.4) to get the peptide-modified electrode.

Electrochemical measurements

The peptide-modified electrode was immersed in 100 μL of 10 mM PB (pH 7.4) containing fixed concentration of trypsin for 35 min, followed by a thorough washing with 10 mM PB (pH 7.4). The electrochemical measurement was performed with SWV in degassed 1.0 mL of 10 mM Tris-HCl (pH 7.4) containing 500 μM $[Ru(NH_3)_6]^{3+}$. The concentration of trypsin was quantified by an increased reduction peak current ($\Delta I = I_s - I_0$), where I_0 and I_S were the reduction peak current of the peptide-modified electrode before and after incubation with trypsin, respectively. All experiments were carried out at room temperature.

Results and discussion

Characterization of the peptide-modified electrode

The fabrication process of the peptide-modified electrode was firstly characterized by cyclic voltammetry and electrochemical impedance spectroscopy in the presence of $[Ru(NH_3)_6]^{3+}$ as redox probe. As shown in Fig. 2a a, the anodic and cathodic

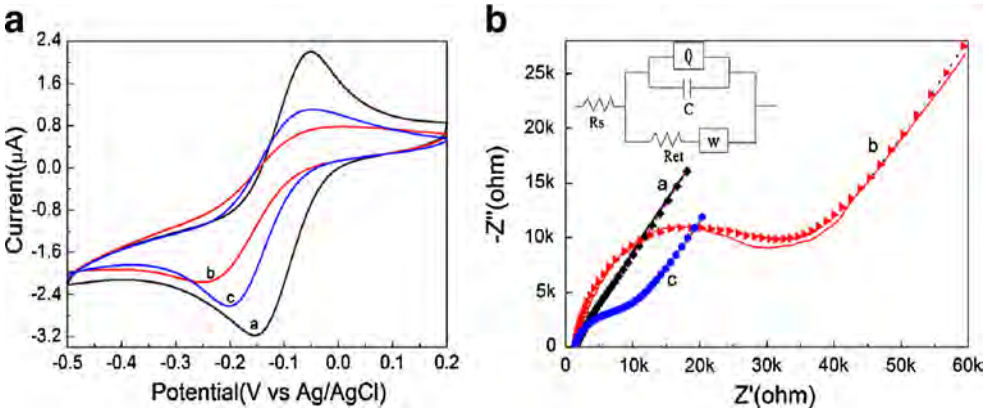

Fig. 2 Cyclic voltammograms **a** and Nyquist plots of impedance spectra **b** of $[Ru(NH_3)_6]^{3+}$ in 10 mM Tris-HCl (pH 7.4) at bare gold electrode **a**; the peptide-modified electrode **b** and the peptide-modified electrode after incubation with 0.052 U.mL^{-1} trypsin **c. a** Scan rate, 50 mV.s^{-1}; **b** The biased potential was −0.10 V vs Ag/AgCl; the frequency was from 10 kHz to 100 mHz and the amplitude was 5 mV (Insert: the Equivalent electrical circuit of Nyquist plot)

peaks of $[Ru(NH_3)_6]^{3+}$ appeared at -54 mV and -154 mV, respectively, with a peak-to-peak separation (ΔEp) of 100 mV, and the reduction peak current of $[Ru(NH_3)_6]^{3+}$ is 3.0 μA at bare gold electrode. The observed ΔEp of ~ 100 mV at the bare gold electrode, larger than the expected value for Nerstian behavior for one electron transfer reversible peak separation, 59 mV, can be attributed to the high uncompensated resistance (R ~ 1,510 Ω). The cathodic/anodic current ratio was 0.95, indicating that a Nernstian wave for $[Ru(NH_3)_6]^{3+}$ at bare gold electrode [17]. At the peptide-modified electrode (Fig. 2a, b), the reduction peak current of $[Ru(NH_3)_6]^{3+}$ decreased from 3.0 μA to 2.15 μA and the peak potential separation ΔE increased from 100 mV to 210 mV. This is mainly attributed to the fact that the positive charged peptide immobilized on the surface of the electrode prohibits the transfer of positively charged redox couple on the electrode. Compared line b with line c in Fig. 2a, it can be clearly observed that the reduction peak current increases from 2.15 μA to 2.64 μA after the peptide-modified electrode reacted with 0.052 U.mL^{-1} trypsin. EIS of $[Ru(NH_3)_6]^{3+}$ at the bare gold electrode showed a nearly straight line (Fig. 2b, a, electron transfer resistance (Ret) =543.2 Ω), indicating a very fast electron transfer process of $[Ru(NH_3)_6]^{3+}$ at a bare gold electrode. When peptide and MCH were immobilized onto the surface of gold electrode, the R_{et} increased to 32.6 KΩ. It may be attributed to electrostatic repulsion between positive charged peptide immobilized on electrode surface and $[Ru(NH_3)_6]^{3+}$ in solution. After the peptide-modified electrode was incubated with trypsin, the R_{et} decreased to 7,868 Ω (Fig. 2 b, c). These results indicate that when trypsin is present, it specifically cleaves the specific peptide, led the peptide to leave the electrode, and resulted in the increase of the electrochemical CV signal and the decease of the EIS signal. The effective cleavage of peptide provides a promising and simple method for the determination of trypsin.

Figure 3 shows AFM images of the peptide-modified gold plate before and after incubation with trypsin. It can be seen that peptide was adsorbed onto the gold plate as randomly oriented monolayers (a) with rms=1.39±0.08 nm. Upon incubation with trypsin, the surface was fairly smooth and the

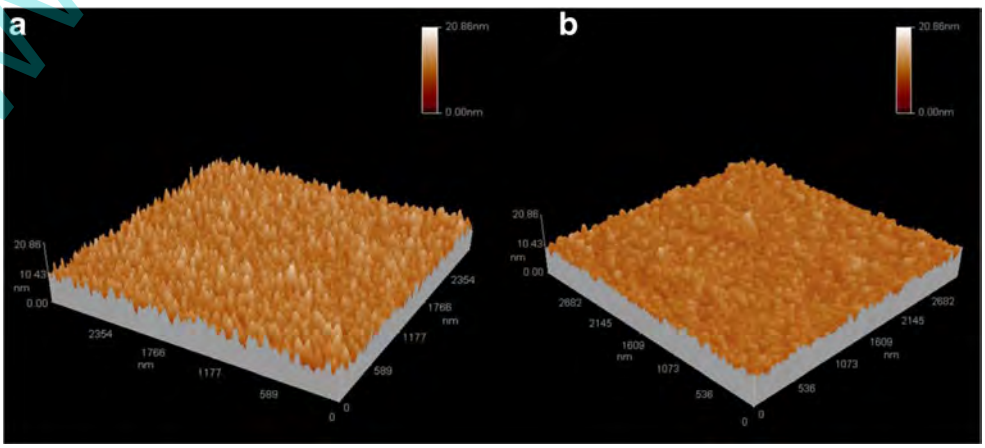

Fig. 3 **a** AFM image of the peptide-modified sputtered gold substrate **b** AFM image of the peptide-modified sputtered gold substrate after incubation with 0.052 U.mL^{-1}trypsin

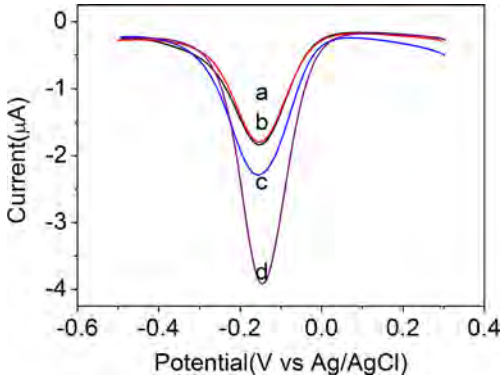

Fig. 4 Square wave voltammograms of 500 μM $[Ru(NH_3)_6]^{3+}$ in 10 mM Tris-HCl (pH 7.4). The peptide modified electrode **a** the peptide modified electrode after incubation with 0.01 % BSA **b**, 0.0047 $U.mL^{-1}$ trypsin **c** and 0.052 $U.mL^{-1}$ trypsin **d** Cleavage time, 35 min. SWV were performed with pulse amplitude of 25 mV and frequency of 15 Hz

rms values decreased to 0.92±0.11 nm (b). These results further indicate that trypsin can cleave the specific peptide.

Here, square wave voltammetry was also used as detect method because of the advantages of being a scanning voltammetric technique (such as CV) enabling quick analyses, low consumption of target species, and reduced fouling of the electrode surface. Moreover, SWV offers a better discrimination of the charging and background currents and better definition of the response than CV [18]. Fig. 4 shows the SWVs of the peptide-modified electrode before and after reaction with trypsin and BSA, respectively. Compared line a and line c, it can be seen that the peptide-modified electrode shows a lower electrochemical reduction current (line a, 1.79×10^{-6} A) while the peptide-modified electrode displays a higher current (line c, 2.29×10^{-6} A) after reaction with 0.0047 $U.mL^{-1}$ trypsin. Compared line c with line d, it can be clearly observed that the peak current increases from 2.29×10^{-6} A to 3.92×10^{-6} A as the concentration of trypsin is elevated from 0.0047 –0.052 $U.mL^{-1}$. A negligibly low signal is observed when 0.01 % BSA (line b, 1.83×10^{-6} A) is used for the test. The results indicate that the electrochemical method is feasible for the determination of trypsin. Compared the change of reduction peak current in Fig. 4 with that in Fig. 2a, a high sensitivity was obtained when using SWV as detection technique. Therefore, SWV was used for the quantitative assay of trypsin in the following experiment.

Optimization of conditions

In electrochemical method, the immobilization of a substrate on the surface of a transducer plays an important role because the immobilization of peptide onto electrode not only produces a functionalized sensing interface but also determines the sensitivity, selectivity and reproducibility of the biosensor [19]. Here, the peptide was immobilized on the surface of a gold electrode through a self-assembled process. It was found that the electrochemical response of the peptide-modified electrode was strongly affected by the probe density of the substrate peptide on electrode, which is related to the self-

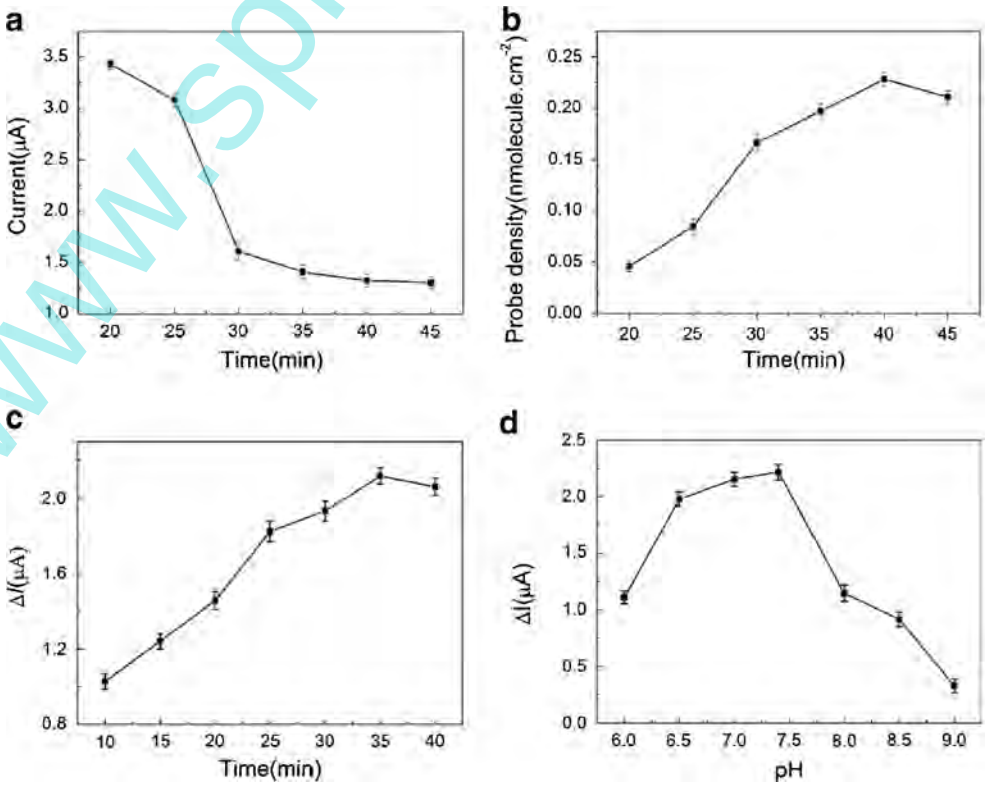

Fig. 5 **a** Dependence of the peak current of the peptide-modified electrode on self-assembly time; **b** Dependence of the probe density of the peptide-modified electrode on self-assembly time; **c** Dependence of the increased peak current on cleavage time for 0.052 $U.mL^{-1}$ trypsin; **d** Dependence of the increased peak current on pH for 0.052 $U.mL^{-1}$ trypsin. Measurement conditions are the same as those in Fig. 4

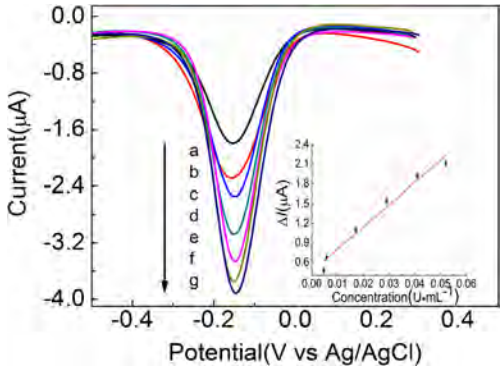

Fig. 6 SWVs responses to different concentrations of trypsin. **a** 0 U.mL^{-1}, **b** 0.0047 U.mL^{-1}, **c** 0.0058 U.mL^{-1}, **d** 0.018 U.mL^{-1}, **e** 0.029 U.mL^{-1} **f** 0.041 U.mL^{-1}, **g** 0.052 U.mL^{-1}. Insert, calibration curve of trypsin. Measurement conditions are the same as those in Fig. 4

assembly time of the probe onto the gold electrode and the concentration of the substrate peptide used. Therefore, the self-assembly time was optimized at fixed concentration of the substrate peptide (10 μg.mL^{-1} peptide). Fig. 5a shows the effect of self-assembly time of on the peak current of the peptide-modified electrode. From Fig. 5a, it can be seen that peak current decreases with increasing self-assembly time from 20 to 40 min and reaches a minimum at 40 min. This is attributed to the fact that the amount of positive charged peptide increases with the self-assembly time and then prohibits the transfer of positively charged redox couple on the electrode. The amount of peptide can be estimated with the charge associated with the electrode desorption reaction arising from the one-electron reduction of peptide layer on gold surface according Faraday law [20]. The packing densities of the peptide, related with the amount of peptide and the electrode surface area (0.038 mm^2 for bare gold electrode), were increased from 4.59×10^{-11} to 2.2×10^{-10} molecules cm^{-2} with the increase of self-assembly time. The maximum peptide density at the gold electrode was 2.2×10^{-10} molecules.cm^{-2} when the self-assembly time is 40 min (Fig. 5b). Therefore, the self-assembly time of 40 min was employed for 10 μg.mL^{-1} peptide to obtain a low background and maximum amount of peptide on the electrode surface for the electrochemical method.

The kinetics of the cleavage reaction catalyzed by trypsin was studied, through stopping the reaction at different time intervals and measuring the electrochemical signal. Figure 5c displays the dependence of the increased peak current (ΔI) on cleavage time between peptide and 0.052 U.mL^{-1} trypsin. The increased reduction peak current sharply increased with increase of cleavage time from 10 min to 35 min. After 10 min of trypsin incubation, 75 % of the ratio of the increased current ($\Delta I/I_0$) observed for the peptide-modified electrode. And the enzymatic digestion of peptide was continued up to 35 min, inducing a progressive current increase ratio up to 148 % from the initial current response. A slight decrease was obtained at 40 min. The increase of reduction peak current indicates that the specific proteolytic cleavage event of peptide on the electrode surface in the presence of 0.052 U.mL^{-1} trypsin is completed within 35 min at room temperature. The cleavage time is longer than it was found in [6, 10 min] obtained with colorimetric method but shorter than that in [5, 2 h] although the same method was used, and is longer than it was found in [8, 20 min] obtained with fluorescence method but shorter than that in [7, 24 h] although the same method was used. The cleavage time is nearly same as in [9, 15 min] and [10, 30 min] obtained with electrochemical method. The different cleavage time may be related the origination of enzyme and the detection model. To ensure the completeness of cleavage between peptide and trypsin in this work, 35 min was set as the cleavage time in following experiments.

The effect of pH of trypsin solution on the increased reduction peak current is examined using different pH buffer containing 0.052 U.mL^{-1} trypsin. It was found that the increased peak current were dependent on the buffer pH and the maximum response was obtained between pH 6.5 and 7.4 (Fig. 5d). Therefore, a pH 7.4 phosphate buffer was used to prepare trypsin solution.

Analytical performance for the determination of trypsin

The quantitative behavior of the electrochemical method was assessed under the optimized conditions. Figure 6 shows

Table 1 Analytical performance for the determination of trypsin

Method	Substrate	Linear range	Detection limit	Refs
Colorimetric	gelled protein	0.50~100 μg.mL^{-1}	0.5 μg.mL^{-1}	5
Colorimetric	peptide	/	1.6 ng.mL^{-1}	6
Fluorescence	cytochrome c	0~1 μM	33 ng.mL^{-1}	7
10~400 μM				
Fluorescence	peptide	0~20 ng.mL^{-1}	0.25 ng.mL^{-1}	8
Electrochemistry	peptide	/	2 mU.mL^{-1}	9
Electrochemistry	gelatin	1~250 ng.mL^{-1}	1 ng.mL^{-1}	10
Electrochemistry	gelatin	0.75~7,500 U.mL^{-1} (0.1~1,000 μg. mL^{-1})	0.01 μg.mL^{-1} (0.075 U mL^{-1})	11
Electrochemistry	peptide	0.0047~0.052 U.mL^{-1} (0.2~2 ng.mL^{-1})	0.0012 U.mL^{-1} (5 ng.mL^{-1})	This work

SWV responses to different concentrations of trypsin. From Fig. 6, it is clearly seen that the electrochemical reduction peak current gradually increases when the activity of trypsin is elevated from 0.0047 to 0.052 U.mL^{-1}. The increased peak current ΔI is directly proportional to the trypsin activity in the range from 0.0047 to 0.052 U.mL^{-1} (Fig. 6, insert). The linear regression equation is $\Delta I = 4.7\times10^{-7}\ C + 3.4\times10^{-5}$ (unit of C is U.mL^{-1}) with the correlation coefficient of 0.9718. The detection limit is calculated to be 0.0012 U.mL^{-1} (5 ng.mL^{-1}) trypsin (3σ) [21]. The results were listed in Table 1. An immunoassay-based, quantitative study found healthy individuals to have a mean serum trypsin concentration of 0.25±0.1 μg.mL^{-1}, whereas acute pancreatitis patients exhibited a higher concentration of 1.4±0.6 μg.mL^{-1} [22]. From Table 1, it can be seen that the sensitivity is evident and the detection limit of this method is sufficiently low for detection of pathological conditions. The relative standard derivation (RSD) for 0.0058 U.mL^{-1} trypsin was 3.2 % (n=5). The peptide-modified electrode stored at 4 °C in 10 mM PB (pH 7.4) for 15 days shows that the reduction peak current for the detection of 0.0058 U.mL^{-1} trypsin is 88 % of initial reduction peak current. This result indicates that an acceptable reproducibility and storage stability of the peptide-modified electrode is feasible.

An evaluation of the selectivity of the electrochemical method was performed by examining 2.3×10^{-8} g.mL^{-1} (0.0058 U.mL^{-1}) trypsin or 1.0×10^{-6} g.mL^{-1} other proteins, including thrombin, GR and AchE, respectively. The increased reduction peak current for 0.0058 U.mL^{-1} trypsin (7.8×10^{-7}A) is much bigger than that for nearly 50-fold higher level (1.0×10^{-6} g.mL^{-1}) of thrombin (1.6×10^{-7} A), GR (1.5×10^{-7} A) and AchE (1.3×10^{-7}A), respectively. The results indicate that the peptide substance has negligible activity with these enzymes. The small response for the three enzymes may be ascribed to the defect of self-assembly monolayer or the thiol desorption [23]. A good selectivity of the electrochemical method for the determination of trypsin is evident.

Conclusion

Here, we develop a simple and sensitive electrochemical method for the determination of trypsin by employing a specific heptapeptide (CRRRRRR) as a substrate. This method does not require tedious and complicated labeling and analytical procedures, which provides a general and promising platform for the detection of enzyme cleavage event. This strategy presented could be easily extended to monitor the activity of other kinases.

Acknowledgments We gratefully acknowledge the financial support from The National Science Foundation of China (no. 21375084) and the Natural Science Basic Research Plan in Shaanxi Province of China (nos. 2014JQ2065, 2013KJXX-73, 2013SZS08-Z01 and 2013SZS08-P01) and the Fundamental Research Funds for the Central Universities (no. GK201302050).

References

1. Miao P, Liu T, Li X, Ning L, Yin J, Han K (2013) Highly sensitive, label-free colorimetric assay of trypsin using silver nanoparticles. Biosens Bioelectron 49:20–24
2. Hirota M, Ohmuraya M, Baba H (2006) The role of trypsin, trypsin inhibitor, and trypsin receptor in the onset and aggravation of pancreatitis. J Gastroenterol 41(9):832–836
3. Temler RS, Felber JP (1976) Radioimmunoassay of human plasma trypsin. Biochim Biophys Acta 445(3):720–728
4. Rhodes MB, Hill RM, Feeney RE (1957) Spectrophotometric determination of trypsin and trypsin inhibitors. Anal Chem 29(3):376–378
5. Zaccheo BA, Crooks RM (2011) Self-powered sensor for naked-eye detection of serum trypsin. Anal Chem 83(4):1185–1188
6. Xue W, Zhang G, Zhang D (2011) A sensitive colorimetric label-free assay for trypsin and inhibitor screening with gold nanoparticles. Analyst 136:3136–3141
7. Li X, Zhu S, Xu B, Ma K, Zhang J, Yang B, Tian W (2013) Self-assembled graphene quantum dots induced by cytochrome c: a novel biosensor for trypsin with remarkable fluorescence enhancement. Nanoscale 5(17):7776–7779
8. Fan H, Jiang X, Zhang T, Jin Q (2012) Peptide-induced fluorescence quenching of conjugated polyelectrolyte for label-free, ultrasensitive and selective assay of protease activity. Biosens Bioelectron 34:221–226
9. Abd-Rabboh HSM, Nevins SA, Dürüst N, Meyerhoff ME (2003) Electrochemical assay of protease activities based on polycation/polyanion complex as substrate and polyion sensitive membrane electrode detection. Biosens Bioelectron 18:229–236
10. Ionescu RE, Cosnier S, Marks RS (2006) Protease amperometric sensor. Anal Chem 78:6327–6331
11. Stoytcheva M, Zlatev R, Cosnier S, Arredondo M (2012) Square wave voltammetric determination of trypsin activity. Electrochim Acta 76:43–47
12. Arredondo M, Stoytcheva M, Zlatev R, Gochev V (2012) Some clinical applications of the electrochemical biosensors. Mini Rev Med Chem 12(12):1301–1313
13. An L, Tang Y, Feng F, He F, Wang S (2007) Water-soluble conjugated polymers for continuous and sensitive fluorescence assays for phosphatase and peptidase. J Mater Chem 17:4147–4152
14. Nigatu AS, Vupputuri S, Flynn N, Neely BJ, Ramsey JD (2013) Evaluation of cell-penetrating peptide/adenovirus particles for transduction of CAR-negative cells. J Pharm Sci 102(6):1981–1993
15. Love JC, Estroff LA, Kriebel JK, Nuzzo RG, Whitesides GM (2005) Self-assembled monolayers of thiolates on metals as a form of nanotechnology. Chem Rev 105(4):1103–1169
16. Carvalhal RF, Freire RS, Kubota LT (2005) Polycrystalline gold electrodes: a comparative study of pretreatment procedures used for cleaning and thiol self-assembly monolayer formation. Electroanalysis 17:1251–1259
17. Bard AJ, Faulkner LR (2000) Electrochemical methods, fundamentals and applications, 2nd edn. Wiley, INC
18. Gonzalez J, Molina A, Ortiz FM, Laborda E (2012) Characterization of the electrocatalytic response of monolayer-modified electrodes with square-wave voltammetry. J Phys Chem C 116:11206–11215

19. Rao SV, Anderson KW, Bachas LG (1998) Oriented immobilization of proteins. Microchim Acta 128(3–4):127–143
20. El-Deab MS, Ohsaka T (2004) Molecular-level design of binary self-assembled monolayers on polycrystalline gold electrodes. Electrochim Acta 49:2189–2194
21. Kaiser H (1973) Guiding concepts relating to trace analysis. Pure Appl Chem 34:35–62
22. Artigas JM, Garcia ME, Faure MR, Gimeno AM (1981) Serum trypsin levels in acute pancreatic and non-pancreatic abdominal conditions. Postgrad Med J 57:219–222
23. Kahsar KR, Schwartz DK, Medlin JW (2012) Liquid- and vapor-phase hydrogenation of 1-epoxy-3-butene using self-assembled monolayer coated palladium and platinum catalysts. Appl Catal A Gen 445–446:102–106