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Facile electrochemical detection of botulinum neurotoxin type E using a two-step proteolytic cleavage

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ABSTRACT

Facile electrochemical methods for measuring protease concentration or protease activity are essential for point-of-care testing of toxic proteases. However, electrochemical detection of proteases, such as botulinum neurotoxin type E (BoNT/E), that cleave a peptide bond between two specific amino acid residues is challenging. This study reports a facile and sensitive electrochemical method for BoNT/E detection. The method is based on a two-step proteolytic cleavage using a target BoNT/E light chain (BoNT/E-LC) and an externally supplemented exopeptidase, L-leucine-aminopeptidase (LAP). BoNT/E-LC cleaves a peptide bond between arginine and isoleucine in IDTQNRQIDRI-4-amino-1-naphthol (oligopeptide-AN) to generate isoleucine-AN. Subsequently, LAP cleaves a bond between isoleucine and AN to liberate a free electroactive AN species. The liberated AN participates in electrochemical–chemical–chemical (ECC) redox cycling involving $Ru(NH_3)_6^{3+}$, AN, and a reducing agent, which allows a high signal amplification. Electrochemical detection is carried out without surface modification of indium – tin oxide electrodes. We show that dithiothreitol is beneficial for enhancing the enzymatic activity of BoNT/E-LC and also for achieving a fast ECC redox cycling. An incubation temperature of 37 °C and the use of phosphate buffered saline (PBS) buffer resulted in optimal signal-to-background ratios for efficient BoNT/E detection. BoNT/E-LC could be detected at concentrations of approximately 2.0 pg/mL, 0.2, and 3 ng/mL after 4 h, 2 h, and 15 min incubation in PBS buffer, respectively, and approximately 0.3 ng/mL after 2-h incubation in bottled water. The method developed could be applied in fast, sensitive, and selective detection of any protease that cleaves a peptide bond between two specific amino acid residues.

1. Introduction

Proteases play a crucial role in digesting proteins by cleaving peptide bonds and thereby control protein activity. Some proteases that exist in microbes and viruses act as toxins and are hazardous to humans and animals. For this reason, rapid and accurate methods for measuring protease concentration or protease activity have been developed. Based on the number of specific amino acid residues that are involved in proteolysis, proteases can be classified into two types: (i) proteases that cleave a peptide bond before or after a specific amino acid residue and (ii) proteases that cleave a peptide bond between two specific amino acid residues. The first type of proteases can liberate a label moiety (connected to a specific amino acid residue) that is capable of generating a colorimetric (Suaifan et al., 2013), fluorescent (Hu et al., 2012), or electrochemical signal (Park and Yang, 2014), which has been widely employed for protease detection. However, this cannot be applied for detection of the second type of proteases mainly because more than one amino acid residue remains attached to the label after the proteolysis reaction. For detection of the second type of proteases, (i) the change in fluorescence intensity caused by fluorescence resonance energy transfer (FRET) between donor and acceptor labels located at two terminals of an oligopeptide and (ii) the change in electrochemical signal of an electroactive label caused by the cleavage of an oligopeptide attached to an electrode that occurs after proteolysis, have been used. However, such methods require that the oligopeptide be modified with two labels (Li et al., 2011) or with an electroactive label followed by attachment to an electrode (Lowe et al., 2012). To overcome this, it is necessary to develop a simpler method for detecting the second type of proteases.

Botulinum neurotoxins (BoNTs) are a high-molecular-weight protein with 3 components: a heavy chain, a light chain, and a

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nontoxic hemagglutinin (Zhang et al., 2012). Because the heavy chain permits the toxin to bind with the cell, and the light chain contains zinc-dependent endopeptidase, the BoNTs excluding a heavy chain or light chain are not hazardous to human. BoNTs are classified into several serotypes. Among them, BoNT types A, B, and C are most fatal biological toxins that cause botulism (Anderson, 2012). Infection with poisonous BoNTs produces agonizing symptoms like nerve palsy and paralysis of voluntary muscles, which may lead to death. Moreover, the rapid wide spread of botulism possibly owing to bioterrorism can cause an urgent public health threat (Zhang et al., 2012; Anderson, 2012). Thus, it is essential to determine the serotype and activity of BoNTs rapidly and sensitively. In several cases, BoNT detection is performed by immunoassays that rely on the specific binding between BoNT (antigen) and an antibody (Kirsch et al., 2013; Stanker et al., 2008). However, such immunoassays lack necessary specificity to determine the BoNT serotype (Scarlatos et al., 2005). For this purpose, methods that are based on the specific proteolytic activity of BoNTs have been commonly used to determine the serotype. In these methods, the detection signal is based on principles of fluorescence (Grate et al., 2009), chemiluminescence (Cadieux et al., 2005), electrochemiluminescence (Cheng and Stanker, 2013), surface plasmon resonance (Homola, 2003), or mass spectrometry (Kalb et al., 2010).

Electrochemical detection has been primarily considered for point-of-care testing of proteases, because it allows fast and sensitive detection with a small, simple instrument (Akanda et al., 2014). Although electrochemical immunoassays for BoNTs and electrochemical detection based on impedance spectroscopy and the proteolytic reaction of BoNTs (Ye et al., 2013) have been developed, electrochemical detection based on redox reaction of an electroactive species generated by the proteolytic activity of BoNTs has never been reported. This may be due to the fact that BoNTs cleave a peptide bond between two specific amino acid residues and that proteolytic cleavage of an electrochemical label-connected oligopeptide leaves more than one amino acid residue next to the label (Li et al., 2000). Therefore, the use of an additional protease to liberate the label becomes necessary. Compared to endopeptidases like BoNTs, exopeptidases cleave a terminal amino acid residue of a peptide. Aminopeptidases and carboxypeptidases are types of exopeptidases that specifically cleave an amino acid residue at the *N*-terminal and *C*-terminal, respectively (Burley et al., 1991; Lipscomb, 1970).

In this study, we report an electrochemical method for rapid, simple, and sensitive detection of the light chain of BoNT type E (BoNT/E-LC) that cleaves a peptide bond between two specific amino acid residues. Detection is based on a two-step proteolytic cleavage using a target BoNT/E-LC and an externally supplemented exopeptidase L-leucine-aminopeptidase (LAP). Proteolytic cleavage liberates an electroactive 4-amino-1-naphthol (AN) moiety that participates in electrochemical-chemical-chemical (ECC) redox cycling to obtain an amplified electrochemical signal without surface modification of an electrode. In the first part of this study, the specific proteolytic activity of LAP for a bond between isoleucine and AN is investigated. Optimum conditions for two-step cleavage are also investigated. In the latter part, BoNT/E-LC in phosphate buffered saline (PBS) buffer and commercial bottled water is detected and detection limits are calculated.

2. Materials and methods

2.1. Materials

C-terminally AN-labeled oligopeptide (IDTQNRQIDRI) [oligopeptide-AN] and L-isoleucine-*p*-nitroanilide (Ile-NA) were obtained from Anygen Co., Ltd. (Jangseong-gun, Korea). LAP from porcine kidney (EC 3.4.11.1), AN, $[Ru(NH_3)_6]Cl_3$, tris(2-carboxyethyl)phosphine (TCEP), dithiothreitol (DTT), PBS, 4-(2-hydroxyethy1)-1-piperazineethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, and $ZnCl_2$ were obtained from Sigma-Aldrich Co. Indium–tin oxide (ITO) electrodes were obtained from Corning Co. (Daegu, Korea). Commercial bottled water (Jeju Samdasoo) was purchased from Kwang Dong Pharmaceutical Co. (Seoul, Korea). The bottled water contains 2.5 – 4.0 mg/L Ca^{2+} and 1.7 – 3.5 mg/L Mg^{2+}. Buckwheat water and tea (rooibos) were purchased from Sulloc Co. (Seoul, Korea), and coffee (Maxim Kanu) was purchased from Dongsuh Co. (Seoul, Korea).

2.2. Preparation of BoNT/E-LC

cDNA encoding BoNT/E-LC (1–422 a.a.) was synthesized from Genscript (U.S.A) and cloned into a modified pET28a vector generating *N*-terminal His-tag with TEV protease site between the His-tag and the protein. BoNT/E-LC was expressed in BL21 in the presence of 10 μM $ZnCl_2$ with 1 mM isopropyl-β-D-1-thiogalactopyranoside for 18 h. The cells were resuspended with Tris buffer (50 mM, pH 8.0) containing 300 mM NaCl and 5% (v/v) glycerol and lysed by ultrasonication. The cell debris was removed by centrifugation at 14,000 rpm for 1 h. BoNT/E-LC were purified with Ni-NTA (Qiagen) affinity chromatography. The His-tag was removed by TEV protease and the uncleaved BoNT/E-LC was removed by Ni-NTA. BoNT/E-LC was further purified by Superdex S200 (GE healthcare) size-exclusion gel chromatography equilibrated with Tris buffer (50 mM, pH 8.0) containing 100 mM NaCl.

2.3. Procedure for BoNT/E-LC detection

0.5 mM oligopeptide-AN, 5.0 mM $Ru(NH_3)_6^{3+}$, and 10.0 mM DTT (or TCEP) were prepared in four different buffers, i.e., PBS buffer (pH 7.4), Tris buffer (50 mM, pH 7.4), Tris buffer (50 mM, pH 7.4) containing 1.0 mM $ZnCl_2$, and HEPES buffer (20 mM, pH 7.4). For detecting BoNT/E-LC in PBS buffer and bottled water, 250 μL of a solution containing different concentrations of BoNT/E-LC was mixed with 250 μL of a buffer solution containing 0.5 mM oligopeptide-AN. To this, 250 μL PBS buffer containing 10 μg/mL LAP and 250 μL buffer containing 10.0 mM DTT (or TCEP) were mixed. The mixture was incubated at 25 or 37 °C for either 15 min, 2 h, or 4 h. Subsequently, 250 μL of a buffer solution containing 5.0 mM $Ru(NH_3)_6^{3+}$ was added. The resulting solution was injected into an electrochemical cell, and electrochemical data were recorded after an incubation for two-step proteolytic cleavage. The final concentrations of LAP, $Ru(NH_3)_6^{3+}$, DTT (or TCEP), and oligopeptide-AN were 2 μg/mL, 1.0 mM, 2.0 mM, and 0.1 mM, respectively.

2.4. Electrochemical measurement

ITO electrode substrates (1 cm × 2 cm) were pretreated with a solution of 5:1:1 H_2O: (30%) H_2O_2: NH_4OH at 70 °C for 1 h (Choi et al., 2013). A Teflon electrochemical cell was assembled using an ITO electrode, an Ag/AgCl (3 M NaCl) reference electrode, and a platinum counter electrode. The exposed geometric area of each ITO electrode was approximately 0.28 cm^2. Chronocoulometry was performed at room temperature using a CHI 1040C (CH Instruments, Austin, TX, USA).

2.5. Absorbance measurement

1 mL PBS buffer containing 1.66 mM L-isoleucine-*p*-nitroanilide (Ile-NA) or 1.66 mM Ile-NA and 2.5 mM DTT (or TCEP) was mixed with 35 μL PBS buffer containing 1.0 U/mL LAP, or 35 μL Tris buffer