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Evaluation of Two Types of Biosensors for Immunoassay of Botulinum Toxin

Kibong Choi,* Wonjun Seo, Seunghee Cha, and Jungdo Choi†

GWSD-4-3, Agency for Defense Development, P.O. Box 35, Yusung, Taejon 305-600, Korea

† Department of Biochemistry, Chungbuk National University, Cheongju 361-763, Korea

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Immunoassay of botulinum toxin (BTX) B type was investigated using two types of biosensors: light addressable potentiometric sensor (LAPS) and surface plasmon resonance (SPR) sensor. Urease-tagged and immuno-filtration capture method have been used for LAPS. Tag-free and direct binding real-time detection method have been used for SPR sensor. The detection limit of sandwich assay format with LAPS was 10 ng/ml, which was the lowest among methods tested. SPR has the advantage of being more convenient because tag-free direct binding assay can be used and reaction time was reduced, regardless of low sensitivity. This result shows that sandwich assay format with LAPS can be used as an alternative method of BTX mouse bioassay which is known as the most sensitive method for the detection of BTX.

Keywords: Biosensor, Botulinium toxin, Light addressable potentiometric sensor, Surface plasmon resonance sensor

Introduction

Botulinum toxin (BTX) is a high-molecular-weight protein that affects practically all vertebrates. Seven immunological types, A through G, of the toxin which are highly related to their structure and function have been found. The macrostructure of this neurotoxin was known that the single-chain molecule undergoes a postsynthetic cleavage of nicking by a protease, such as trypsin or chymotrypsin, endogenous or exogeneous to the bacterium. BTX is separated into two fragments, designated as the heavy chain (BTX·HC, 100KD) and the light chain (BTX·LC, 50KD), by reduction of disulfide bond (Sakaguchi *et al.*, 1988).

* To whom correspondence should be addressed. Tel: 82-42-821-4669; Fax: 82-42-821-2391; Email: bong525@chollian.net

The most sensitive and widely used method for detecting BTX is the intraperitoneal injection of samples of serum or food extracts into mouse that weight 18–22 g. This method has many drawbacks. For example, it takes 4 days to perform and requires large number of mice. Furthermore the results fluctuate according to the difference of experimental animals and adminstration methods (Notormans and Nagel, 1989). Immunoassay, such as enzyme-linked immunosorbent assay (Elisa), was suggested as an alternative to mouse bioassay. However, the sensitivity of this assay was not enough to replace the mouse assay, which is known to be 5–10 pg/ml, or 0.03–0.07 pM (Doellgast *et al.*, 1994).

In recent development of bioanalytical devices, especially biosensors, several attractive features of potentialities such as selectivity, sensitivity and size of equipment are emphasized (Byfield and Abuknesha, 1994). For novel biosensor, surface plasmon resonance (SPR), resonant mirror (RM) and light addressable potentiometric sensor (LAPS) were used as signal transducer (Lukosz, 1991; Bousse and Parce, 1994; Watts *et al.*, 1994). LAPS is a silicon-based sensor, which was recently developed as a highly sensitive device for measuring pH, redox potentials, or transmembrane potentials from biochemical reactions.

Biosensors utilizing a biospecific binding reaction are often based on surface physical phenomena occurred when biomolecules interact on a solid surface. SPR sensor is based on the measurement of changes in the refractive index of the immediate vicinity of a metal surface. With these two types of biosensors, LAPS and SPR, we investigated the sensitivity profile of BTX detection with monoclonal antibodies that can recognize the heavy- or light-chain of BTX, B type neurotoxin. In particular, we focused on sensitivity enhancement and comparison of two biosensors by changing the immunoassay format and optimizing reaction conditions of the two biosensors, aiming at the replacement of current BTX mouse bioassay.

Materials and Methods

Reagents and Equipments Dinitrophenol-biotin-N-hyrdoxysuccinimide (Biotin-NHS), carboxyfluorescein-N-hydroxysuccinimide (Fluorescein-NHS), streptavidin, anti-fluorescein-IgG-urease, urea and biotinylated nitrocellulose membrane (0.45 μm pore) were from Molecular Devices (Sunnyvale, California, USA). N-Hydroxysuccinimide (NHS), N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and ethanolamine were from Biacore AB company. Other reagents were purchased from Sigma chemical company (St. Louis, MO, USA).

Botulinum toxin type B and their monoclonal antibodies were donated from the Department of Biological Science of Korea Advanced Institute of Science and Technology. These antibodies were able to recognize the heavy chain and light chain of the toxin, respectively.

As a bioanalytical device, LAPS which was manufactured by Molecular Devices company (Sunnyvale, California, USA) and commercially available as the Threshold Unit of the trade name, was used. BIAcore X, as the sensor of SPR, and sensor chip CM-5 were from BIAcore AB Co. (Uppsala, Sweden). This device is equipped with an autoinjector, microfluidic system and software package for system control and evaluation of the data. Sensor chip CM-5 was modified with carboxymethyl dextran matrix on thin gold film of the chip.

Labeling of BTX and monoclonal antibodies for LAPS BTX and monoclonal antibody against light chain of BTX (anti-BTX·LC) were labeled with fluorescein. To 1 ml of the BTX solution (0.25 mg/ml) or antibody solutions (0.25 mg/ml) in 10 mM phosphate buffered saline (PBS) was added 4.34 μl of fluorescein-NHS solution (4.2 mg/ml) in anhydrous dimethylformamide. This is to make the initial molar ratio of fluorescein to protein to be 20 : 1. After 2 h of incubation, the unbound fluorescein-NHS and fluorescein-labeled protein were separated with the elution of PBS solution through the desalting column.

Antibody against heavy chain of BTX (anti-BTX·HC) was labeled with biotin. To 1 ml of antibody solution (0.18 mg/ml) in 10 mM PBS was added 3.27 μl of biotin-NHS solution (5 mg/ml) in anhydrous dimethylformamide, in order to make the initial molar ratio of biotin to protein to be 20 : 1. After 2 h of incubation, the unbound biotin-NHS and biotin-labeled antibody were separated with the elution of PBS solution through the desalting column.

Competitive immunoassay using LAPS Immunoassay was done: (a) adding 100 μl of BTX solution with various concentrations in assay buffer (10 mM PBS, pH 7.4, 0.05% Triton X-100) to mixed solution of 50 μl of biotin-labeled anti-BTX·HC solution and 50 μl of fluorescein-labeled BTX solution in assay buffer, (b) incubation of the mixture for 2 h at room temperature, (c) adding 1 ml of streptavidin solution (1 μg/ml) in assay buffer, (d) filtration on biotinylated nitrocellulose membrane at 100 μg/min to bound conjugate onto biotinylated membrane, (e) washing with 2 ml of wash buffer (10 mM PBS, pH 6.5, 0.05% Tween-20) at 200 μl/min to remove unbound streptavidin, (f) adding 1 ml of anti-fluorescein-IgG-urease (1 μg/ml) in assay buffer, (g) washing with 2 ml of wash buffer at 200 μl/min, (h) inserting the membrane into detection chamber of threshold unit filled with 100 mM urea solution in wash buffer as a substrate for signal generation, (i) attaching the membrane to the sensor surface with plunger, and (j) measuring LAPS signal as μV/sec unit.

Sandwich immunoassay using LAPS One hundred μl of BTX, 50 μl of fluorescein-labeled anti-BTX·LC and 50 μl of biotin-labeled anti-BTX·HC were mixed. After 2 h of incubation, LAPS signal was measured as the same procedure for competitive assay.

Antibody immobilization onto SPR sensor chip The same volume of NHS (11.5 mg/ml) and EDC (75 mg/ml) were mixed together, and then 35 μl of the mixed solution was injected into the device for the activation of carboxymethyl dextran on surface of CM-5 chip. The flow rate and temperature were adjusted to 5 μl/min and 25°C, respectively, throughout the procedures of the activation and the immobilization. Into the activated sensor chip was injected 35 μl of anti-BTX·LC (75 μg/ml) or anti-BTX·HC (75 μg/ml) in 10 mM HEPES buffered saline (pH 7.4). Residual surface which was unbound with antibody was deactivated with 35 μl of 1 M ethanolamine solution (pH 8.5).

Quantitation of botulinum toxin using SPR sensor After the injection of 80 μl of BTX with various concentrations in HEPES buffered saline (pH 7.4, 10 mM) into the device, the increase of SPR signal was measured. After each measurement, sensor chip was regenerated by washing with 10 μl of 0.1 mM HCl solution.

Results and Discussion

Competitive immunoassay using LAPS Fluorescein-labeled BTX (50 ng or 100 ng) and biotin-labeled anti-BTX·HC (100 μl) with various concentrations were mixed. After 2 h of incubation, LAPS signal were measured as shown in Fig. 1. The signal with 100 ng of fluorescein-labeled BTX was twice compared to that with 50 ng fluorescein-labeled BTX. Thus optimum concentration of fluorescein-labeled BTX was decided as 100 ng in the competitive assay format. Saturation effect of biotin-labeled anti-BTX·HC began to exhibit from the concentration of 50 ng/100 μl. Thus, the starting concentration of biotin-labeled anti-BTX·HC was decided as 50 ng in the competitive assay format.

Based on the above optimal conditions of fluorescein-labeled BTX (100 ng/100 μl) and biotin-labeled anti-BTX·HC (50 ng/100 μl), competitive assay of BTX was carried out with 100 μl of varying concentrations of BTX. LAPS signals of assay after 3 h of incubation are shown in Fig. 2. The LAPS signal was decreased as the concentration of unlabeled BTX was increased because of the competition of fluorescein-labeled BTX and unlabeled BTX for the anti-BTX·HC on membrane. The detection limit calculated from the following definition of detection limit was 250 ng/ml.

$$X_{antigen}\text{-}2SD_{antigen} > X_0 + 2SD_{X0}$$

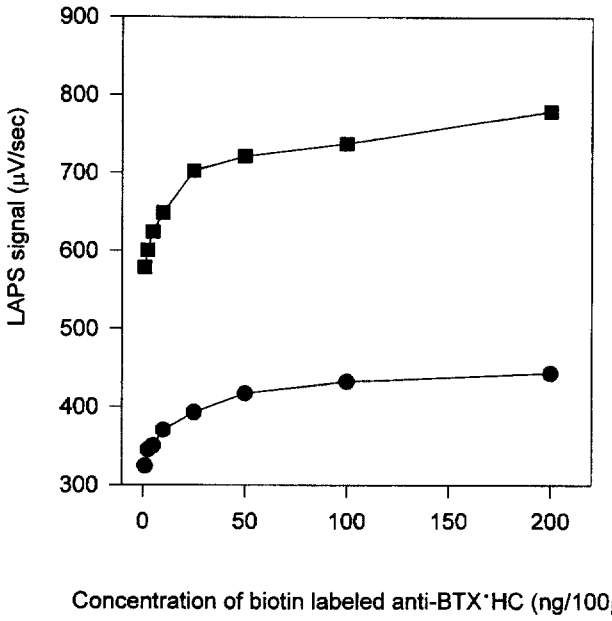

Fig. 1. Determination of optimum concentration of fluorescein-labeled BTX and biotin-labeled anti-BTX·HC for competitive immunoassay using LAPS. ● 50 ng of fluroescein-labeled BTX, ■ 100 ng of fluorescein-labeled BTX.

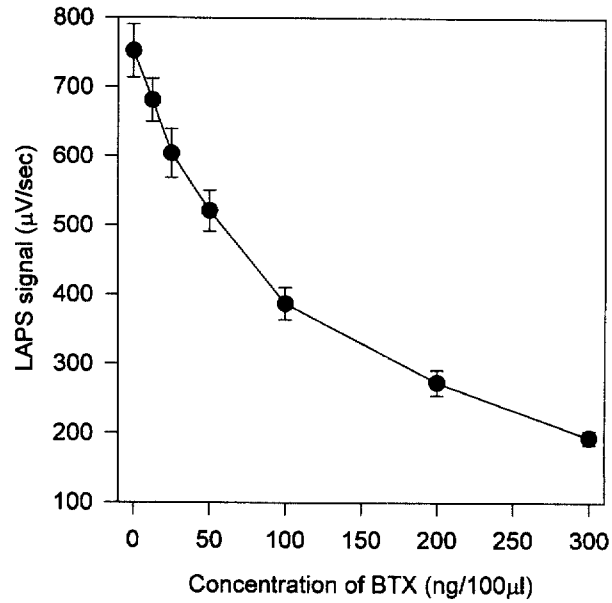

Fig. 2. Detection profile of BTX for competitive immunoassay using LAPS with fluorescein-labeled BTS (100 ng) and biotin-labeled anti-BTX·HC (50 ng).

where, $X_{antigen}$ = mean antigen signal
X_0 = mean background signal
SD = standard deviation

Sandwich immunoassay using LAPS Equal volume (100 μl) of BTX (100 ng/100 μl), fluorescein-labeled anti-BTX·LC (25 ng or 50 ng/100 μl) and biotin-labeled anti-BTX·HC with varying concentration in PBS were mixed. After 2 h of incubation at room temperature, LAPS signal were measured as shown in Fig. 3. To optimize signal generation in sandwich immunoassay using LAPS, 50 ng of fluorescein-labeled anti-BTX·LC was selected because its signal was twice higher compared to 25 ng. As the amounts of biotin-labeled anti-BTX·HC increase, the LAPS signal also increased proportionally. However saturation was observed from 50 ng per 100 μl. From this data, 50 ng of biotin-labeled anti-BTX·HC was chosen as the concentration of initial reaction in next BTX-sandwich immunoassays.

Various concentrations of 100 μl of BTX in assay buffer were mixed with 100 μl of fluorescein-labeled anti-BTX·LC (50 ng per 100 μl) and 100 μl of biotin-labeled anti-BTX·HC (50 ng per 100 μl) in PBS. The LAPS signal against concentration of BTX was plotted in Fig. 4. LAPS signal increased proportionally to the increase of concentration of BTX. Detection limit of this sandwich immunoassay was 10 ng/ml. This is 25 times lower than that of the above competitive immunoassay. The major reason for this difference was thought to be a large standard deviation of the large LAPS signal. The LAPS signals were small at low concentration of BTX in sandwich assay, while those were large in competitive assay.

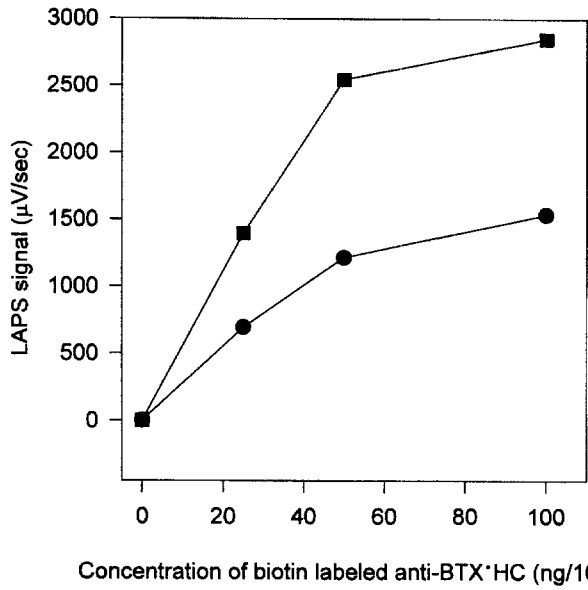

Fig. 3. Determination of optimum concentration of fluorescein-labeled anti-BTX·LC and biotin-labeled anti-BTX·HC for sandwich immunoassay using LAPS. ● 25 ng of fluorescein-labeled BTX·LC, ■ 100 ng of fluorescein-labeled BTX·HC.

In the previous study, the detection limit of mouse IgG assay using polyclonal anti-mouse IgG in LAPS was 156 pg/ml. Thus, detection limit of BTX is about 64 times higher than that of mouse IgG. Anti-mouse IgG has many

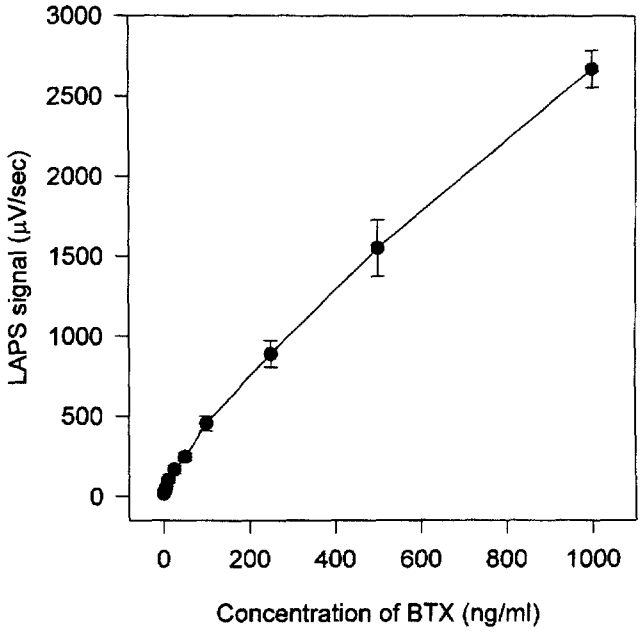

Fig. 4. Detection profile of BTX for sandwich immunoassay using LAPS with fluorescein-labeled BTX·LC (50 mg) and biotin-labeled anti-BTX·HC (50 ng).

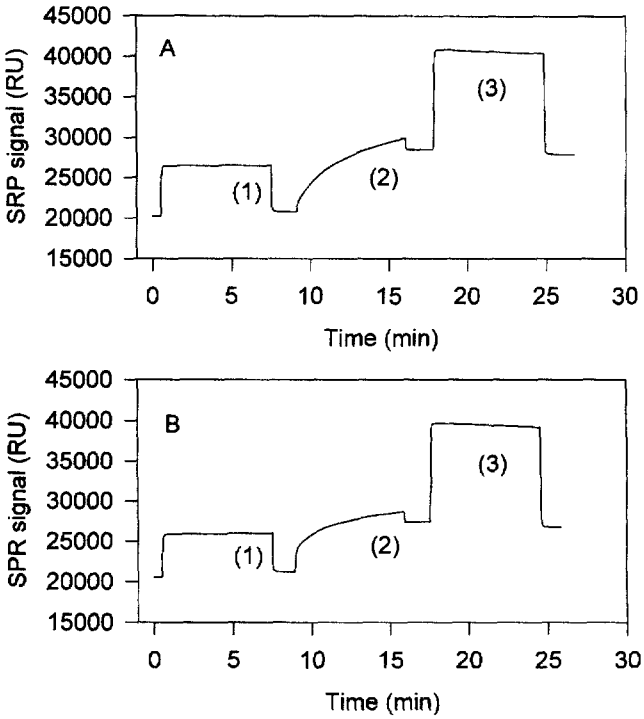

Fig. 5. The sensorgram of SPR sensor showing the sequential procedures and responses of the immobilization of antibodies against BTX heavy chain (A) and light chain (B). (1) Activation of carboxymethyldextran with mixture of NHS and EDC. (2) Immobilization of anti-BTX antibodies. (3) Deactivation of activatedcarboxymethyldextran with ehtanolamine.

antigenic determinant for binding with polyclonal anti-mouse IgG, while BTX has only one antigenic determinant for monoclonal anti-BTX·HC and anti-BTX·LC. This indicates that one mouse IgG can bind with many biotin-anti-mouse IgG and fluorescein-anti-mouse IgG, resulting in larger LAPS signal, while one BTX can bind with only one of each monoclonal anti-BTX·HC and anti-BTX·LC. In addition, low affinity of monoclonal anti-BTXs may be another reason, that is, the number of anti-mouse IgG cannot exceed 10 due to large molecular weight of IgG, although mouse IgG can bind with many polyclonal anti-mouse IgG.

Immunoassay using SPR sensor The sensorgram in Fig. 5 shows the profile of immobilization of anti-BTX·HC and anti-BTX·LC through amine coupling on the CM-5 sensor chip. Resonance unit (RU) increased by anti-BTX·HC (7715.1 RU) was higher by 25% than that by anti-BTX·LC (6178.5 RU). According to the technical manual of BIAcore X, SPR detection area is 0.224 mm^2 (1.4 × 0.16 mm) and 1000 RU is corresponded to about 1 ng/mm^2 of the immobilized biomass. Thus, total amounts of the immobilized anti-BTX·HC and anti-BTX·LC can be calculated as 1.72 ng (7715.1 RU × 0.224 × 1 ng/mm^2·1000 RU) and 1.38 ng (7715.1 RU × 0.224 × 1 ng//mm^2·1000 RU), respectively.

Analytical profile of BTX (80 μl in HBS, pH 7.4, 5 μl/min of flow rate) with various concentrations using SPR chip immobilized with anti-BTX·HC or anti-BTX·LC is shown in Fig. 6. In both cases, SPR signal

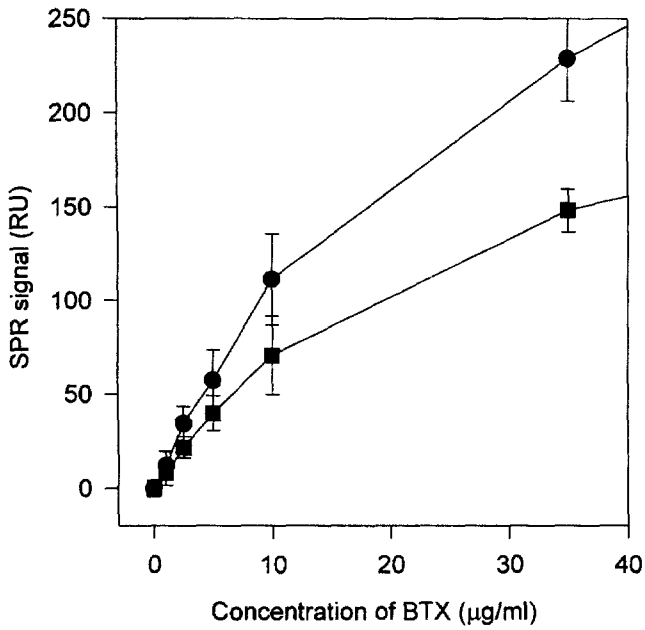

Fig. 6. Detection profile for direct binding assay of BTX using SPR sensor chip coated with anti-BTX·HC (●) and anti-BTX·LC (■) in SPR sensor. The values are means ±S.E. (n = 4) of LAPS signal in μN/sec.

increased linearly up to 10 μg/ml of the concentration of BTX and their slope were reduced in higher concentration. Detection limit of BTX in this SPR binding assay was 2.5 μg/ml. The value of detection limit in SPR is 10 or 250 times less sensitive than that of competitive immunoassay or that of sandwich immunoassay in LAPS, respectively. The SPR signal with anti-BTX·HC was higher by 60% than that with anti-BTX·LC immobilized chip. It was thought that these differences came from the amount of immobilized antibody. The amounts of immobilized anti-BTX·HC and anti-BTX·LC in sensing area were calculated as 1.72 ng and 1.38 ng, respectively. This is because of the higher affinity of anti-BTX·HC compared with anti-BTX·LC.

The detection limit of BTX in SPR assay was 2.5 μg/ml and injected volume was 80 μl. Thus minimum amount of BTX required in SPR assay may be 200 ng. The height of flow cell is 50 μm, and the height of carboxymethyl dextran layer in the assay in 150 mM salt concentration is 100 nm. Assuming the laminar flow in the flow cell, the amount of BTX introduced into carboxymethyl dextran layer was 0.4 ng (200 ng × 100 nm per 50000 nm) which corresponds to 333.3 RU. The measured increment of resonance unit with anti-BTX·HC and anti-BTX·LC were 21.9 and 34.4, respectively, which are about one-tenth of theoretical values. But the theoretical values are underestimated because the stream in flowing cell is not laminar. Actually, the amount of BTX introduced into carboxymethyl dextran layer is over 0.4 ng because mixing of solution occurred in the flow channel. Thus the ratio of bound BTX to unbound BTX should be over 10. If we can reduce this ratio, larger LAPS signal will be obtained. Total reaction time of the assay calculated from the flow rate (5 μl/min) and the volume of BTX solution (80 μl) was 16 min. In usual immunoassays including Elisa and LAPS, time for reaction between antigen and antibody is 2 h or more. Our results indicate that the detection limit of BTX in SPR sensor could be lowered by increasing incubation time or controlling reaction condition.

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