Anal Bioanal Chem (2011) 400:2865–2869
DOI 10.1007/s00216-011-5019-y

SHORT COMMUNICATION

Antibody characterization and immunoassays for palytoxin using an SPR biosensor

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Received: 28 February 2011 / Revised: 11 April 2011 / Accepted: 12 April 2011 / Published online: 27 April 2011

Abstract Palytoxin (PLTX), a polyether marine toxin originally isolated from the zoanthid *Palythoa toxica*, is one of the most toxic non-protein substances known. Fatal poisonings have been linked to ingestion of PLTX-contaminated seafood, and effects in humans have been associated with dermal and inhalational exposure to PLTX containing organisms and waters. Additionally, PLTX co-occurrence with other well-characterized seafood toxins (e.g., ciguatoxins, saxitoxins, tetrodotoxin) has hindered direct associations of PLTX to seafood-borne illnesses. There are currently no validated methods for the quantitative detection of PLTX(s). As such, a well-characterized, robust, specific analytical technique is needed for the detection of PLTX(s) in source organisms, surrounding waters, and clinical samples. Surface plasmon resonance (SPR) biosensors are ideally suited for antibody characterization and quantitative immunoassay detection. Herein, we describe a newly developed SPR assay for PLTX. An anti-mouse substrate was used to characterize the kinetic values for a previously developed monoclonal anti-PLTX. The characterized antibody was then incorporated into a sensitive, rapid, and selective PLTX assay. Buffer type, flow rate, analyte-binding time, and regeneration conditions were optimized for the antibody–PLTX system. Cross-reactivity to potentially co-occurring seafood toxins was also evaluated. We show that this optimized assay is capable of measuring low- to sub-ng/mL PLTX levels in buffer and two seafood matrices (grouper and clam). Preliminary results indicate that this SPR biosensor assay allows for (1) rapid characterization of antibodies and (2) rapid, sensitive PLTX concentration determination in seafood matrices. Method development information contained herein may be broadly applied to future PLTX detection and/or antibody characterization efforts.

Keywords Palytoxin · Surface plasmon resonance · Biosensor · Antibody characterization · Immunoassay

Abbreviations

EFSA	European Food Safety Authority
HBS-EP+	10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% (*v*/*v*) surfactant P20 buffer
k_a	Association rate constant
K_D	Dissociation constant
k_d	Dissociation rate constant
PBS	10 mM phosphate-buffered saline pH 7.4
PLTX	Palytoxin
R_{max}	Analyte-binding capacity
RU	Resonance unit
SPR	Surface plasmon resonance
t_c	Mass transfer constant (units $RU \times M^{-1}s^{-2/3}m^{-1/3}$)
U	Uniqueness value
χ^2	Average-squared residual

Introduction

Palytoxins (PLTXs) are a group of complex, extremely potent, marine natural products first described from tropical

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Cnidarian zoanthids [1]. With a mouse lethality (IP LD_{50}) of 300 ng/kg, they are one of the most potent non-protein natural products discovered to date [2]. Unique among the marine biotoxins, PLTXs represent a risk to humans through ingestion, inhalation, and dermal routes [3, 4]. Their distribution, toxicology, mode of action, and methods of detection have all been recently reviewed [5–8]. Currently, there are no regulations for the control of PLTX(s) in seafood, but the European Food Safety Authority (EFSA) has recently recommended a limit of 30 μg/kg shellfish meat [9].

Since their first discovery, numerous assays and analyses have been used to detect PLTX(s) (for review see [7]). These methods vary greatly in their sensitivity and specificity. For regulatory purposes, a combination of screening methods with analyte-specific chemical confirmatory methods (e.g., LC-MS) may be required. We describe here a surface plasmon resonance (SPR) biosensor that holds the potential to be a sensitive, reliable, and quantitative screening method for PLTX.

Several antibodies specific for PLTX have been developed over the years [10–12] with immunoassays and in vitro hemolysis neutralization assays developed from them [13, 14]. SPR biosensors offer the unique capability of antibody characterization and then incorporation of these ligands into rapid, sensitive assays on the same platform. SPR technology has been used extensively in the pharmaceutical arena, as well as in food safety. In fact, assays for toxins already exist, including those for the marine toxin tetrodotoxin [15]. While the tetrodotoxin assay is in an inhibition format, SPR biosensors can also measure the specific interaction of a surface-bound recognition element (e.g., antibody) with a target analyte (e.g., toxin).

In this research, we first determined the kinetic properties of a previously developed antibody for PLTX. The characterized antibody was then used in a direct detection assay for PLTX spiked into buffer and two seafood matrices (grouper and clam). The assay was optimized for the following parameters: buffer type, flow rate, binding time, and regeneration conditions. In addition, antibody cross-reactivity was evaluated. These studies elucidate the antibody kinetics for an anti-PLTX antibody and show that a rapid, sensitive (low- to sub-ppb), and selective assay for PLTX can be designed using the characterized antibody.

Experimental

Materials

A Biacore T100 instrument was used for all analyses (GE Healthcare, Piscataway, NJ, USA). Associated materials, including CM5 series S chips, amine coupling kit, mouse antibody capture kit, regeneration solutions, and HBS-EP+, were also obtained from GE Healthcare. Grouper and clam extracts were prepared by adding 20 mL of 80% ethanol to 5 g of homogenized tissue. These suspensions were vortexed and then centrifuged at 4,000 rpm for 10 min. For compatibility with instrument fluidics, the 80% ethanolic supernatant extracts were diluted 10× with HBS-EP+.

PLTX, 10 μg/mL in 50% ethanol, was used as the stock for all standard solutions. PLTX (2,680 Da from *Palythoa tuberculosa*) was purchased from Wako Pure Chemicals Industries, Ltd. (Japan). The mouse monoclonal anti-PLTX production has been previously described [10]. The batch used herein was produced 21 August 2007 at the US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD. Phosphate-buffered saline (PBS; 10 mM, pH 7.4) was purchased from Sigma Aldrich (St. Louis, MO, USA). Millipore Milli-Q 18.2 MΩ·cm water (Billerica, MA, USA) was used for all solution preparations. Saxitoxin (FDA reference standard, 100 μg/mL), tetrodotoxin (Sankyo Co., Tokyo, Japan, 10 mg/mL), maitotoxin (generous gift from Professor Takeshi Yasumoto, 2 μg/mL), pectenotoxin (NRC, Ottawa, Canada, 0.429 μg/mL), okadaic acid (NRC, 2 μg/mL), and dinophysistoxin 1 (Wako Pure Chemicals Industries, 0.5 μg/mL) were also acquired.

Surface plasmon resonance biosensor

A direct assay for PLTX was performed for both kinetic measurements and concentration assays. For all experiments, flow cell one was a reference cell (only ethanolamine), whereas flow cells two through four were active (anti-PLTX).

Antibody characterization

For kinetic studies, a capture approach was used for the sensor chip formation. Following standard protocol [16], a mouse antibody capture surface was prepared using covalent coupling. This surface allows the capture of mouse anti-PLTX and facile regeneration (removal of the anti-PLTX from the anti-mouse surface). This approach allows for fresh antibody, in an optimum orientation, and at a consistent surface density (~1,100 resonance units (RU)) for each measurement of antibody–PLTX interaction. In contrast to surfaces created for concentration assays, this capture approach significantly lowers antibody surface density thus reducing the potential for multiple-binding events that would impact apparent kinetics. This capability is an advantageous feature of SPR assays, as more accurate determinations of affinity can be achieved (versus avidity measurements commonly attained when using ELISA).

Kinetic assays were performed by first injecting mouse anti-PLTX (50 μg/mL) over the anti-mouse surface at 10 μL/min for 150 s. PLTX was then introduced with a contact time of 120 s (50 μL/min) followed by a 180-s dissociation period. Finally, the surface was regenerated via 10 mM glycine–HCl, pH 1.7 (180 s, 30 μL/min), and the assay repeated at numerous PLTX concentrations.

Immunoassay

For concentration assays, the direct capture of anti-PLTX to the CM5 surface is preferable due to a higher surface ligand density and, in turn, better sensitivity. Preconcentration studies showed that 50 μg anti-PLTX/mL in 10 mM sodium acetate buffer, pH 4.5 was best for antibody conjugation. Direct capture of anti-PLTX proceeded via standard amine coupling, and ~9,750 RU of antibody was immobilized in flow cells two through four. The sensor chip was conditioned with 3× 30-s pulses of 10 mM glycine–HCl, pH 2.0 and 2× startup cycles of HBS-EP+. The chip was then used for assay optimization and generation of standard curves. Assay conditions of running buffer, flow rate (5, 15, 30, 50, and 75 μL/min), binding time (60, 90, and 120 s), and regeneration solution (10 mM glycine: pH 3.0, 2.5, and 2.0) were evaluated. In addition, cross-reactivity studies were performed with saxitoxin, tetrodotoxin, maitotoxin, pectenotoxin, okadaic acid, and dinophysistoxin 1. All calibration curve data were fit with GraphPad Prism 5 (La Jolla, CA, USA).

Results and discussion

Antibody characterization

Figure 1 shows representative sensorgrams and modeled fit for each curve for five PLTX concentrations. In this data, PLTX injection occurs at 0 s, followed by the direct association of PLTX with the surface-bound anti-PLTX. Upon transitioning back to buffer flow (120 s), the PLTX dissociates with respect to the binding affinity. As the concentration of PLTX in the sample solution increases, the signal (RU) from the direct binding of PLTX to the anti-PLTX also increases. This data set has the parameters of low concentration PLTX yielding linear association curves, while the highest concentration nearly reaches a steady state; these features are essential to reliable determination of kinetic constants. A 1:1 binding model (e.g., one PLTX molecule binding to a single site on the anti-PLTX) with local R_{max} fit (e.g., modeled, maximum analyte binding for each curve due to small variability in ligand activity for each regenerated anti-PLTX surface) was performed using the Biacore T100 Evaluation Software (Table 1).

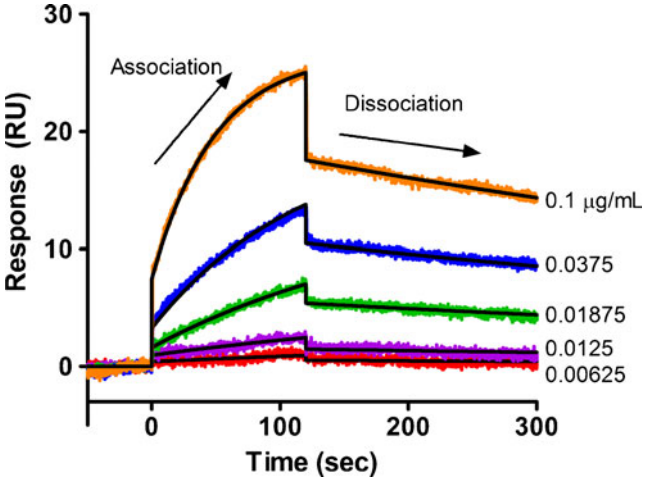

Fig. 1 Sensorgrams for a series of PLTX concentrations (μg/mL, *colored curves*) with the modeled fit to each curve as an overlaid *black line*. Data fit to a 1:1 binding model with local R_{max}; injection spikes removed

The association (k_a) and dissociation rates (k_d) both show low standard error in measurement, and the dissociation constant (K_D) has a value of 2.14×10^{-9} M which follows with the general range of 10^{-7} to 10^{-10} M for monoclonal antibody affinities. In addition to determining that the values are biologically reasonable, the mathematical significance was assessed as follows. All values fell within expected tolerances of no mass transport (t_c) challenges, had uniquely determined rate constants (U value), showed reasonable theoretical maximum response (R_{max}), and had low average-squared residuals (χ^2) of the modeled points to the experimental values indicating that the determined K_D is realistic.

Immunoassay

Initial assays using the characterized antibody focused on optimization of running conditions. First, the running buffer was evaluated. HBS-EP+ and PBS allowed for equal binding of PLTX to the anti-PLTX surface. As HBS-EP+ is known to decrease potential non-specific binding to the chip surface and instrument fluidics, this buffer was chosen for the remainder of the studies. Next, the flow rate was tested to determine if there were mass transfer constraints. No mass transfer limitations were seen, and a flow rate of 50 μL/min gave reasonable signals for all concentrations studied. A contact time of 120 s with a 15-s dissociation time was chosen for PLTX injections. The best regeneration solution (most efficient removal of PLTX from the sensor without damage to anti-PLTX) was 10 mM glycine, pH 2.0 with a 45-s contact time (30 μL/min).

Cross-reactivity studies were also performed with potentially co-occurring seafood toxins: saxitoxin, tetrodotoxin, maitotoxin, pectenotoxin, okadaic acid, and dinophysis-

Table 1 Kinetic assay results for the monoclonal palytoxin antibody

Value	Result	Interpretation
k_a	$5.236\times10^{5}\pm4.9\times10^{3}$ $M^{-1}s^{-1}$	0.9% standard error
k_d	$1.121\times10^{-3}\pm1.0\times10^{-6}$ s^{-1}	0.09% standard error
K_D	2.14×10^{-9} M	Relatively strong monoclonal
t_c	9.014×10^{13}	This high value indicates not mass transport limited
U	4	U<15 indicates parameters are unique (not correlated)
R_{max}	20.26 RU for 0.1 μg/mL	Matches well with experimental steady state
χ^2	0.204	1.01% of R_{max} = Good fit

t_c=mass transfer constant, $RU\times M^{-1}\,s^{-2/3}\,m^{-1/3}$. U=uniqueness value. A software test for pairwise parameter correlation (e.g., k_a and k_d); if correlation is detected, the constants are not uniquely determined (e.g., ratio of constants can be calculated but not absolute values for each). R_{max}=theoretical analyte-binding capacity estimated from the amount of ligand on the surface and the relative sizes of ligand and analyte. Values that are too high may indicate non-specific binding, while those that are too low may indicate loss of ligand activity.

toxin 1. At 0.025 μg/mL, these toxins showed no cross-reactivity with the antibody, as their responses (RU) were not significantly different than blank injections (0 ng/mL PLTX). In addition, each toxin (0.025 μg/mL) was mixed with PLTX (0.05 μg/mL) and was injected over the sensor surface. For each solution, the sensor response was equivalent to that of a 0.05-μg/mL PLTX solution showing that there are no additive/competitive interactions. Several structurally similar PLTX analogs have been described from Cnidarian zoanthids, belonging to the genus *Palythoa*, and several dinoflagellates, belonging to the genus *Ostreopsis* (for review see [7]). None are commercially available and therefore, could not be tested here. Future studies will be required to investigate other PLTX forms and PLTX-like toxins (e.g., ostreocins, ovatoxins, and mascarenotoxins) in order to fully understand their kinetics and reactivity with the described anti-PLTX to determine the ability of this assay to fully protect public health.

After optimization, buffer and two seafood matrices (grouper and clam) were spiked with PLTX to generate standard curves, and the sensor response was evaluated (Fig. 2). Successful assays were achieved with each matrix, with higher standard deviations occurring in the grouper and clam matrices. The limit of detection, determined by the response from a blank (0 μg/mL PLTX) injection plus 3× the standard deviation of the blank, was 0.52, 2.8, and 1.4 ng/mL for HBS-EP+, 10% grouper, and 10% clam, respectively. These results highlight the need for matrix matching for accurate determination of PLTX concentration. Further studies will need to focus on assessing the quantitative capabilities of this assay for unknown (spiked and naturally contaminated) PLTX samples.

Conclusions

An SPR biosensor has been used to evaluate a previously developed PLTX antibody and then develop standard curves for PLTX in buffer and two seafood matrices (grouper and clam). The anti-PLTX showed no cross-reactivity to potentially co-occurring seafood toxins and has strong binding kinetics that allow for a rapid, sensitive assay. The assay with this antibody was optimized and had detection limits near those of current methods (e.g., 5 ng/mL using a cell-based assay [17], 1 pg/mL in hemolysis neutralization assays [13]) and could detect PLTX in HBS-EP+ below the EFSA proposed limit of 30 μg PLTX and ostreocin-D/kg shellfish meat [9] if appropriate extraction efficiency is achievable on shellfish samples. The techniques described herein should be applicable to the future characterization of PLTX antibodies and the further development of rapid, sensitive SPR biosensors for PLTX.

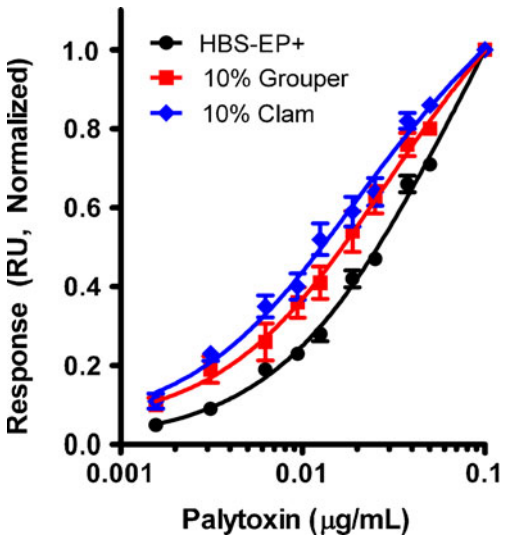

Fig. 2 Calibration curves for PLTX in buffer, grouper, and clam matrices. Data have blank subtraction (to account for bulk refractive index changes) and were normalized to the signal at 0.1 μg/mL PLTX. Each *point* is the average of three measurements

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