Biosensors and Bioelectronics 78 (2016) 111–117

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Simultaneous differentiation and quantification of ricin and agglutinin by an antibody-sandwich surface plasmon resonance sensor

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ARTICLE INFO

Article history:
Received 25 August 2015
Received in revised form
23 October 2015
Accepted 8 November 2015
Available online 11 November 2015

Keywords:
Surface plasmon resonance
Detection
Ricin
Ricinus communis agglutinin
Differentiation
Quantification

ABSTRACT

Ricin is one of the most toxic plant toxins known. Its accessibility and relative ease of preparation makes it a potential agent for criminal or bio-terrorist attacks. Detection of ricin from unknown samples requires differentiation of ricin from the highly homologous *Ricinus communis* agglutinin which is currently not feasible using immunological methods. Here we have developed a simple and sensitive surface plasmon resonance (SPR) sensing system for rapid differentiation between ricin and agglutinin done in real time. Both lectins were quantified in a sandwich immunoassay-like setting by capturing with a cross-reactive antibody (R109) binding to both proteins while differentiating by injection of a ricin-specific antibody (R18) in a subsequent enhancement step. The SPR-assay was reproducible and sensitive for different *R. communis* cultivars, showing no false positive results when other lectins were tested. Quantification and differentiation of both molecules was also demonstrated from a crude castor bean extract and complex matrices. For the first time, we have demonstrated how the closely related lectins can be discerned and quantified in a single assay based on immunological methods. This novel approach delivers crucial information regarding the composition, purity, concentration, and toxicity of suspicious samples containing ricin in less than 30 minutes. Furthermore, we show how enhancement injections during SPR-measurements can be used to determine the ratio of two related proteins independently of the actual protein concentration by comparing normalized enhancement response levels.

1. Introduction

Ricin, which is derived from the seeds of the castor plant *Ricinus communis*, is amongst the most lethal toxins known (Bradberry et al., 2003). The median lethal dose (LD_{50}) is estimated to be 1–10 μg/kg body weight when delivered by inhalation or injection and 1–20 mg/kg body weight when ingested (Worbs et al., 2011). The heterodimeric (A- and B-chain) glycoprotein, with a molecular weight of 60 kDa, belongs to the family of type II ribosome inactivating proteins (Hartley and Lord, 2004). Its high toxicity, availability, and the relative ease of extraction makes ricin a potential agent for bioterrorism (Schieltz et al., 2011). Consequently, ricin is listed as a category B agent of potential bioterrorism risk by the Centers for Disease Control and Prevention (CDC) (Moran, 2002). Moreover, its possession and purification are prohibited by both the Chemical and Biological Weapons Convention (Sidell et al., 1997).

In the past ricin has been used for small scale attacks (Schep et al., 2009) like the assassination of Georgi Markov (Crompton and Gall, 1980). It has also been sent in threat letters to members of the U.S. Senate and the White House (Audi et al., 2005; Hayden and Wadman, 2013). In those cases, rapid, sensitive and ideally unambiguous detection methods are needed to screen for ricin-containing samples.

The detection of ricin is complicated by the fact that, besides ricin, castor seeds contain the homologous, but less toxic, protein *R. communis* agglutinin (Olsnes et al., 1974). Agglutinin is a 120 kDa heterotetrameric protein consisting of two ricin-like heterodimers linked via a disulfide-bond between the two A-chains (Sweeney et al., 1997). Despite a sequence homology of 93% and 84% between the A- and B-chains of ricin and agglutinin (Roberts et al.,

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http://dx.doi.org/10.1016/j.bios.2015.11.020
0956-5663/

1985), ricin is a potent toxin whereas agglutinin is only weakly toxic (Cawley et al., 1978; Saltvedt, 1976). Consequently, discrimination of both proteins is important, as only ricin is recognized as a threat agent. As antibodies are typically unable to differentiate between ricin and agglutinin due to their high sequence identity, the discrimination is technically challenging (Brandon and Hernlem, 2009; Pappenheimer et al., 1974).

To date, only mass spectrometry (MS) based methods allow for differentiation, detection and quantification of both proteins (Fredriksson et al., 2005; Kalb and Barr, 2009; Ma et al., 2014; Tevell Åberg et al., 2013). MS can also be used to combine unambiguous discrimination and identification with functional activity assessment of ricin (Becher et al., 2007; Kalb and Barr, 2009; McGrath et al., 2011; Schieltz et al., 2011, 2015). With a limit of detection (LOD) of 0.64 ng/mL, these methods are highly sensitive, and, by using isotope dilution mass spectrometry, can enable absolute quantification of ricin and agglutinin (McGrath et al., 2011). However, due to the need for sophisticated equipment, and the associated cost and technical expertise, these methods would be preferentially executed in expert laboratories.

Surface plasmon resonance (SPR) based sensors offer the advantage of rapid and label free real-time monitoring of binding events caused by changes of the refractive index near the sensor surface (Fee, 2013; Hodnik and Anderluh, 2009; Larmour and Graham, 2011). For ricin detection, several SPR-sensors have been developed. A portable SPR-sensor based on an antibody-sandwich-setup was able to detect 200 ng/mL ricin within 10 minutes (Feltis et al., 2008). In another study using an monoclonal antibody (mAb) for capture, ricin from seven different horticultural variants could be detected down to 0.5 ng/mL within 15 minutes (Tran et al., 2008). However, differentiation between ricin and agglutinin was not possible. Discrimination of the two proteins was obtained in another work by employing different affinities of ricin and agglutinin towards ß-lactosyl- and ß-galactosyl-ceramides (Uzawa et al., 2008). Using a ß-lactosyl-ceramide functionalized Au sensor surface, 10 pg/mL ricin could be detected within 5 minutes. However, as solely the lectin binding activity of ricin was probed in this assay, problems can be expected in samples containing high concentrations of lactose or galactose, e.g. milk (Rasooly et al., 2012). Additionally, for assessment of the full toxic potential, at least detection of the A- and B-chain is necessary.

To overcome the existing problems in the discrimination and simultaneous quantification of ricin from agglutinin, we established a SPR-based sensor which delivers quantitative results for ricin and agglutinin within a single test. Our goal was to establish a sensor that delivers a level of analysis close to MS-based methods and is applicable in routine laboratories. To address this we build our assay on two mAbs directed against the A- or B-chain of ricin to detect both subunits. Thorough analysis of the antibodies' binding characteristics revealed that both ricin and agglutinin can be detected by the B-chain specific antibody while steric hindrance of the binding of A-chain specific antibody towards agglutinin enabled clear differentiation of both in a second binding step. Moreover, using normalization of the second binding response, the ricin and agglutinin content of mixtures containing both toxins were simultaneously quantified in a single run.

2. Materials and methods

2.1. Antibodies and toxins

Monoclonal antibodies targeting the ricin A- (mAb R18) or B-chain (mAb R109) were produced by standard hybridoma technology as described previously (Pauly et al., 2009). Ricin and agglutinin were purified from seeds of *R. communis* cultivar carmencita. Purity (> 97%) was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and subsequent silver staining, as well as by mass spectrometry (Kull et al., 2010; Worbs et al., accepted manuscript). Purified ricin (> 95%) from *R. communis* cultivars zansibariensis, carmencita, tanzania, gibsonii, sanguineas, india, and impala was kindly provided by U. Pfüller (University Witten/Herdecke, Germany). Purified agglutinins from *Agaricus bisporus*, *Dolichos biflorus*, *Helix pomatia*, and *Triticum vulgaris* were obtained from Sigma-Aldrich.

2.2. SDS-PAGE, colloidal Coomassie-staining, and Silver-staining

Electrophoretic separation of ricin and agglutinin via SDS-PAGE using a Mini-Protean System (Bio-Rad, Munich, Germany) and subsequent colloidal Coomassie-staining or silver staining of separated proteins was performed following standard protocols (Holtzhauer, 1997; Neuhoff et al., 1988; Smith, 1994). For quantification, Coomassie-stained gels were scanned at a ChemiDoc™ MP Imaging System (Bio-Rad) and densitometrical quantification of band intensities was done using the associated software (ImageLab 4.1; Bio-Rad).

2.3. SPR-sensor for differentiation of ricin and agglutinin

Measurements were performed at 25 °C and a Biacore X100 unit (GE Healthcare). HBS-EP+ (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Tween 20, pH 7.4) was used as running buffer supplemented with 10 mg/mL D-galactose (Carl Roth, Karlsruhe, Germany) to suppress unspecific interaction of ricin/agglutinin with immobilized antibodies by competitive inhibition of unspecific lectin binding to N-glycans on the immobilized mAb and the carboxymethyl dextran matrix of the sensor surface.

The B-chain specific anti-ricin mAb R109 (5 μg/mL in 10 mM sodium acetate buffer pH 4.5) was covalently immobilized on flow cell (Fc) 2 of a CM5 sensor chip using standard amine coupling chemistry. An anti-botulinum neurotoxin specific antibody (A1688, Pauly et al., 2009) of the same IgG1 κ isotype as R109 was immobilized on Fc1 as a non-specific negative control antibody.

Samples were injected over the sensor surface at a flow rate of 5 μL/min for 600 s in order to capture both ricin and agglutinin via interaction with mAb R109. After a 300 s injection of running buffer, mAb R18 (10 μg/mL) was injected for 300 s to differentiate between ricin and agglutinin via sandwich formation. The sensor surface was regenerated by a 45 s injection of 10 mM glycine HCl (pH 1.7) at 10 μL/min.

To differentiate between ricin and agglutinin, the binding responses after injection of mAb R18 were normalized to the first binding response caused by ricin/agglutinin binding to mAb R109. By that, responses after R18 binding to ricin were evaluated in relation to the responses of ricin/agglutinin binding to mAb R109, providing proportional binding responses which were independent of the actual toxin concentrations but were, instead, dependent on the ratio of ricin and agglutinin in a given sample.

3. Results

3.1. Analysis of binding properties of the antibodies R18 and R109 towards ricin and agglutinin

We previously established a sandwich ELISA for the analysis of ricin from food and environmental samples (Pauly et al., 2009). The assay utilized an anti-B-chain mAb R109 as capture and an anti-A-chain mAb R18 as detection mAb. Besides the exquisite sensitivity of this assay (2 pg/mL), detection of ricin using these