Biosensors and Bioelectronics 63 (2015) 21–25

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Sensitive and selective detection of thrombin by using a cyclic peptide as affinity ligand

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ARTICLE INFO

Article history:
Received 17 April 2014
Received in revised form
23 June 2014
Accepted 3 July 2014
Available online 10 July 2014

Keywords:
Cyclic peptide
Enzyme reaction
Affinity ligand
Protein detection
Thrombin

ABSTRACT

Here we describe a sensitive assay for thrombin by using a high binding-affinity cyclic peptide against thrombin as affinity ligand. The cyclic peptide is immobilized on the magnetic beads or microplates to selectively capture thrombin. The enriched thrombin then catalyzes the cleavage of a substrate of thrombin to a detectable product. The detection of thrombin is finally achieved by measuring the generated product. This assay enables the detection of thrombin at tens fM in 100 μL of sample solution when fluorogenic substrate was applied, while detection limits reached pM level when chromogenic substrate was used. Thrombin in plasma sample can be detected with this assay. This cyclic peptide affinity ligand shows potentials for thrombin analysis in other detection formats.

1. Introduction

Small peptide ligands that can specifically bind with a target now can be generated from a large library of peptides by using the evolutionary combinatorial techniques, such as mRNA display, phage display, etc. (Borghouts et al., 2005; Brien et al., 2011; James, 2001; Li et al., 2011; Murray and Baliga, 2013; Uzawa et al., 2013), and these peptide ligands are also called peptide aptamers by some researchers. Many peptide ligands have been selected for a variety of targets with high binding affinity and selectivity (Li et al., 2011; Mascini et al., 2012; Murray and Baliga, 2013; Wang and Liu, 2011). These peptide ligands show advantages over immunoantibodies, such as easy generation by in vitro selection, facile production through chemical synthesis, easy introduction of functional groups for labeling and immobilization, low cost in use, and small size, and thus they have great potentials in many applications in biosensors, bioanalysis, therapeutic, and clinic (Borghouts et al., 2005; James, 2001; Li et al., 2011; Mascini et al., 2012; Uzawa et al., 2013). Comparing with the nucleic acid aptamers, the single stranded oligonucleotides specifically binding to a target, which are selected from nucleic acid libraries through systematic evolution of ligands by exponential enrichment (James, 2001; Mascini et al., 2012), the peptide ligands can show comparable binding affinity and selectivity with protein-like feature, and they usually do not possess many negative charges, different from negatively charged nucleic acid ligands. Because 20 different amino acids with various functional groups can be used to build peptide, peptide ligands can involve more diverse interaction formats and conformation than nucleic acid ligand that usually can only use four kinds of nucleotides, which greatly enhance the probability to obtain a peptide ligand for a target.

Thrombin is a multifunctional protease in blood, playing important roles in blood coagulation (Cera, 2008; Huntington, 2012; Popovic et al., 2012). It also participates in many physiological processes, and can be used as a biomarker for some diseases (Maragoudakis and Tsopanoglou, 2009; Kitamoto et al., 2008). The measurement of thrombin is favorable for disease diagnosis, therapy, and biological researches. Many assays for thrombin have been developed in recent years by using nucleic acid aptamers or immunoantibodies (Cho et al., 2009; Citartan et al., 2012; Sassolas et al., 2011). The peptide ligands can be used as substitutes of antibodies or nucleic acid aptamers in biosensing for protein detection (Davis et al., 2007; Johnson et al., 2008; Mascini et al., 2012; Murray et al., 2007; Sano et al., 2006; Song et al., 2011; Temur et al., 2012). Raffler et al. (2003) reported a selected peptide ligand against thrombin with a dissociation constant (K_d) about 166 nM, and we applied this peptide in an assay for thrombin (Zhao and Gao, 2013). Schlippe et al. (2012) recently have successfully selected another novel peptide ligand targeting thrombin with higher binding strength by in vitro selection technique. One of obtained cyclic peptides from Schlippe's group contains 26 amino acids and has the following sequence, MCIIKKSRDPGRCVGSLGHHHHHHRL (Schlippe et al., 2012), in which the two cysteine residues are linked together through disulfide bond. The K_d of this cyclic peptide binding to thrombin

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http://dx.doi.org/10.1016/j.bios.2014.07.010
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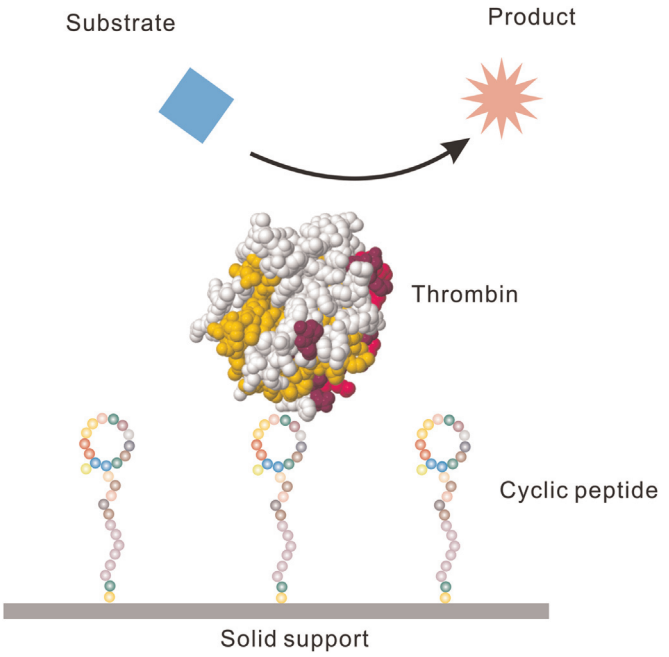

Fig. 1. Schematic diagram of the assay for thrombin relying on the affinity capture with cyclic peptide on solid support and the subsequent enzymatic reaction converting substrate to product.

is around 1.5 nM, 100-fold lower than that of the previously selected small peptide ligand against thrombin by Raffler et al. (2003). Due to the structural rigidity in conformation of cyclic peptide, the cyclic peptide shows higher binding affinity to thrombin than the linear peptide (about 11-fold increase in binding affinity) containing the same sequence, and exhibits a better stability (Schlippe et al., 2012). The application of this emerging cyclic peptide in bioanalysis and biosensing has not been reported and investigated.

Here we aim to describe a simple assay for thrombin with this novel cyclic peptide to demonstrate the analytical application of this cyclic peptide ligand of thrombin by taking advantage of affinity capture and enzyme reaction (Lottenberg et al., 1981; Reymond et al., 2009; Wysocka and Lesner, 2013; Yoon et al., 2012). Fig. 1 shows the schematic principle of the assay using the cyclic peptide ligand following our previous strategy (Zhao et al., 2011, Zhao and Wang, 2012; Zhao and Gao, 2013). The cyclic peptide is conjugated on magnetic beads or microplates, and it specifically captures thrombin from sample solution. The captured thrombin then catalytically converts a fluorogenic or chromogenic substrate to a detectable product. Detection of thrombin is finally achieved by measuring the generated product. This simple assay involves the selective capture of thrombin by cyclic peptide for preconcentration and the efficient and specific enzymatic reaction of thrombin for signal amplification, so thrombin can be detected with high sensitivity and good selectivity. Thrombin at 20 fM in 100 μL of solution was successfully determined without interference from other tested proteins. A wide application of this novel cyclic peptide ligand in bioanalysis and biosensing can be expected.

2. Materials and methods

2.1. Chemical and reagents

Cyclic peptides were synthesized and purified by GL Biochem (Shanghai) Ltd. The used peptide in this work had the following sequence, MCIIKKSRDPGRCVGSLGHHHHHHRLK (Schlippe et al., 2012). The peptide was cyclized through the disulfide bond between two cysteine residues. An additional amino acid, lysine (K), was introduced on the end of the cyclic peptide, and biotin was labeled on the amino acid K for immobilization of the cyclic peptide on solid support. Human alpha thrombin was purchased from Haematologic Technologies Inc. Trypsin (from bovine pancreas), bovine serum albumin (BSA), human serum albumin (HSA), human immunoglobulin G (IgG), and hemoglobin (Hb) from bovine blood were ordered from Sigma. Chymotrypsin and porcine elastase were obtained from Ruibio. Proteinase K was provided by Merck. The fluorogenic substrate of thrombin, N-*p*-tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin hydrochloride, and the chromogenic substrate of thrombin, N-*p*-tosyl-Gly-Pro-Arg-p-nitroanilide acetate were purchased from Sigma. Streptavidin coated magnetic beads (Dynabeads MyOneTM Streptavidin C1) were purchased from Invitrogen Dynal, the magnetic beads had a diameter around 1 μm. The high-binding black 96-well plates (NUNC Maxisorp) were obtained from Thermo Scientific. The high-binding clear 96-well plates were ordered from Corning (Costar 3590). The following buffer solutions were used in assay development, PBS buffer solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5), Tris–HCl buffer solution (20 mM Tris–HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$), and the buffer solution (50 mM Tris–HCl, pH 7.8, 100 mM NaCl, 4 mM $MgCl_2$, 0.25% Triton X-100) used in peptide selection process (Schlippe et al., 2012).

2.2. Preparation of cyclic peptide coated magnetic beads

Streptavidin coated magnetic beads (50 μL, 10 mg/mL) was mixed with 50 μL of biotinylated cyclic peptide (14.6 μM) in PBS buffer solution containing 0.1% Tween 20, and the mixture was incubated at room temperature for 1 h. The cyclic peptide was conjugated on the streptavidin coated magnetic beads through the strong interaction between streptavidin and biotin label. The cyclic peptide modified magnetic beads were washed three times with buffer (PBS buffer + 0.1% Tween 20). Then, they were redispersed into 200 μL of PBS buffer containing 0.1% Tween 20 and 0.1% NaN_3, and stored at 4 °C.

2.3. Preparation of cyclic peptide coated microplates

When fluorogenic substrates were applied in the microplate-based assay for thrombin, a black microplate (NUNC Maxisorp) was used, while a clear plate (Corning, Costar 3590) was used when chromogenic substrates were applied.

The cyclic peptide was conjugated on the microplate by the following procedure. 100 μL of 10 μg/mL streptavidin in coating buffer solution (0.1 M Na_2CO_3, pH 9.6) was added in the well of the plate and incubated at 4 °C overnight, and streptavidin was coated on the surface of the wells. The wells were blocked with the blocking buffer solution (PBS, 2 mg/mL BSA) for 30 min at 37 °C, and then were rinsed with washing buffer solution (PBS + 0.1% Tween 20). After that, 100 μL of biotinylated cyclic peptide at 146 nM was added into the wells of the plate, and incubated for 1 h at room temperature, and biotinylated cyclic peptides were conjugated on the streptavidin coated plates through the strong interaction between biotin and streptavidin. Finally, the plates were washed with PBS solution containing 0.1% Tween 20, and the cyclic peptide coated plates were finally obtained.

2.4. Assays for thrombin using cyclic peptide coated magnetic beads

In the assay for thrombin using cyclic peptide coated magnetic beads, 100 μL of thrombin at varying concentrations were mixed