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Peptide Aptamer-Based ELISA-Like System for Detection of Cathepsin E in Tissues and Plasma

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Abstract

ELISA (enzyme-linked immunosorbent assay), a highly sensitive and powerful molecular detection tool, has been constructed based on antibodies, as its name denotes. However, it is not so easy to prepare antibodies for any target molecule and there are still problems in their cost and stability. In this study, peptide-based ELISA-like systems (*pep-ELISA*) were first constructed and shown to be effective. In particular, if the target is an enzyme, such as cathepsin E, that can generate a fluorescent product, the construct of *pep-ELISA* can be very simple, as its name indicates; i.e., Enzyme-on-Peptide. These constructs, together with peptide-based Sandwich ELISA-like (*Sandwich pep-ELISA*), were actually constructed and examined using the tissues and blood specimens extracted from cathepsin E-normal/knockout rats. Through these experiments, the sufficient sensitivity (~10μg/ml) and methodological convenience of *pep-ELISA* were demonstrated.

Keyword: Peptide aptamer; ELISA-like; Cathepsin E; Diagnosis; Protease

Abbreviations: CatE: Cathepsin E; ELISA: Enzyme-Linked Immunosorbent Assay; pep-ELISA: Peptide Aptamer-Based ELISA-Like Assay; Sandwich pep-ELISA: Peptide-Aptamer-Based Sandwich ELISA-Like Assay; *EOP*: Enzyme on Peptide

Introduction

Highly sensitive detection methods of molecules are essential for scientific and medical purposes: information molecules, such as cytokines and hormones, and possible biomarkers, such as PSA and GAT, are usually in very low concentrations of less than μM and even far less (sub pM or so) [1,2].

Therefore, special techniques like RIA (radio-immunoassay), ELISA (enzyme linked immunosorbent assay) and others have been developed for this purpose [3-6]. Among these, ELISA is the most popular and widely applicable [7,8] because it depends on antibodies and enzymes but not on radio-isotopes, thus being easily manipulated in usual laboratories. Currently, the most sophisticated method for the high sensitivity must be the ELISA system where the antibody plays the pivotal role. Since the diversities of antibodies have been developed and are still expanding [9,10], ELISA has become nearly *de-facto* standard for high sensitivity detection of molecules.

In this paper we demonstrate that peptides can play the role of antibodies in the ELISA system in some cases. This is beneficial since it is difficult to identify desirable antibodies (of high affinity and stability) and costly to prepare antibodies in a large quantity while peptides have been becoming more easily developed by the emerging in vitro evolution method [11-13] than antibodies. At this moment, we have to recall major materials for in vitro evolution, i.e., RNA/DNA aptamers, which have been used for developing various functional molecules such as biosensors [14,15], biomarkers [16], diagnostics [17,18], and therapeutics like Macugen [19-21] and are now increasingly developing owing to its readiness in the molecular evolution [22,23]. Since they have potentially a strong binding affinity to the target molecule at even a pM level of the dissociation constant [24], the RNA/DNA aptamers must be surely choices for developing the substitute for the antibody working for ELISA. However, so far as we know, RNA/DNA aptamer-substituted Sandwich ELISA, a highly specific and useful type of ELISA, has been reportedonce [25] or so probably due to their bulky molecular size (20-30 kD or more) and the conformationally strict requirement [26]. Herein, peptides have appeared as a promising substitute for antibodies. Originally, the peptide has the favorable property that it is far smaller than an antibody (~100 fold) and thus is easy to be synthesized and modified and is less costly. Therefore, it is quite natural to seek away to replace antibodies with peptides in order to set up an ELISA-like system.

In this paper, we report two types of peptide-based ELISA-like systems. One is enzyme-on-peptide (*EOP*), which is much simpler as it consists of a peptide and a target enzyme only. The other looks like the conventional sandwich ELISA where antibodies are replaced with peptides. These were confirmed to be effective using protease cathepsin E as the target.

Materials and Methods

Peptides preparation

The peptides used were cathepsin E (hereafter, CatE) inhibiting/activating peptides that were identified by the *in vitro* evolution method (Table 1) [11].The activities of peptides (pp2 and pp1015 obtained at

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Name	Amino acid sequence (N→C)	Size (a.a.)	Activity (%)	
			A_{dp} [a]	A_{sy} [b]
pp 2	NYKDSCIGGD LTPSSCGGII IISCIA	26	68.9	59.5
pp 1015	PGSSSCIIGG GPGGDGGDPG GPTDSIIIIS RIG	33	162.7	160.7
pa 4043	PGIKPPPCII IIG	13	160±20[c]	-

[a] A_{dp} is the activity of an *in vitro translation* synthesized peptide
[b] A_{sy} is the activity of a chemically synthesized peptide
[c] Under neutral pH conditions

Table 1: Characteristics of peptides used for the pep-ELISA system.

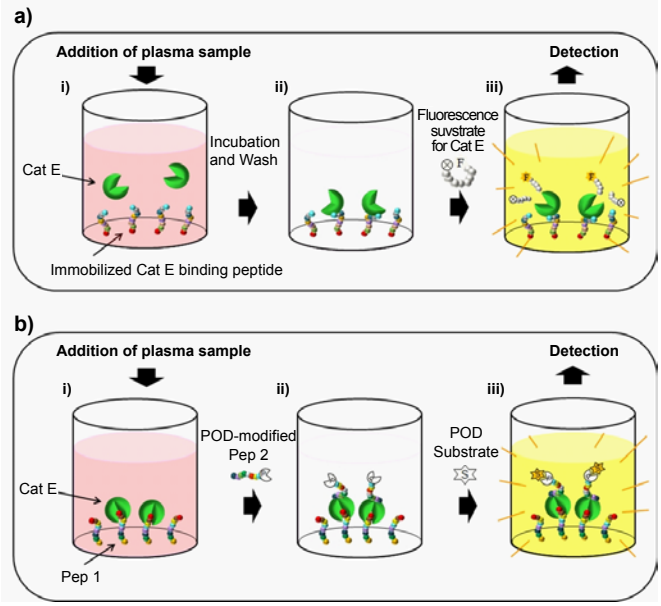

Figure 1: Schematic drawing of peptide-based ELISA-like assay (*pep-ELISA*). a) Enzyme-on-peptide (*EOP*) mode. The target protein (enzyme cathepsin E), trapped by the peptide on the matrix (or a bead), exerts the protease activity, generating fluorescent products. b) Peptide-based Sandwich ELISA-like. The roles of two kinds of antibodies are replaced by two kinds of peptides. The other scheme is quite similar tothe conventional one.

acidic pH (pH 4.5)) were analyzed according to previously reported CatE activity assay with an *in vitro* synthesis-derived peptide and, for further confirmation, with a chemically synthesized peptide [11]. The activity of the peptide p4043, which was obtained as a CatE-activator at neutral pH (pH 7.4),was measured in slightly modified assay conditions using a novel substrate(Nma-Gly-Gly-Arg-Arg-Ser-Gly-Thy-Cys-Gly(Dnp)-D-Arg-NH2)thatcan work at a neutral pH for CatE. In general, CatE was pre-incubated with a peptide aptamer (either inhibitor or activator) in an equal molar ratio at 25°C for 10 min and was combined with a fluorogenic substrate to monitor the enzymatic activity. The CatE-activity (activation/inhibition) was measured by subtracting the background fluorescence of the negative reaction mixture consisting of only the fluoregenic substrate (B_f) from that of the reaction product (C_f forthe control reaction without regulatory peptides or S_f for the sample reaction with regulatory ones). The percentage of inhibition *(I)* or activation *(A)* was calculated by fitting the data to the following equations.

$$I = 100 \times \{1-(S_f - B_f) / (C_f - B_f)\} \quad \text{\% inhibition} \quad (1)$$

$$A = 100 \times (S_f - B_f) / (C_f - B_f) \quad \text{\% activation} \quad (2)$$

These peptides were chemically synthesized by Nihon Bio Service (Scrum Corporation, Japan) and were confirmed by MALDI-TOF-MS.

Peptide immobilization for the Enzyme-on-Peptide (*EOP*)-typed *pep-ELISA*

Peptide molecules were diluted with a Coupling buffer (0.2M $NaHCO_3$, 0.5M NaCl, (pH8.3)) to 1mg/ml, and each of 50µl of the peptide solution was poured into an array of a Peptide coating plate (TaKaRa, Japan). Immediately, 10µl of Reaction solution (TaKaRa) was added into each well and incubated at room temperature for 2h. After incubation, the solutions were moved from the wells of the plate and the peptide-coated wells were washed with 200µl of distilled water three times. Then, 200µl of a Blocking solution (0.5M ethanolamine, 0.5M NaCl (pH8.3)) was added into each well and they were incubated at room temperature for 1 h in order to deprive them of non-specific binders. After removing the Blocking solution, the wells were washed with 200µl of distilled water three times.

Trapping the target enzyme by the cognitive peptide (*EOP*)

First, CatE protease-containing tissue extracts or serum were pH-adjusted with a buffer (500 mM sodium acetate, 1M NaCl, pH 4.0) to pH 4.5.When the precipitation was generated, it was removed by centrifugation at 15,000 rpm for 5 min and the supernatant was recovered. The supernatant of 30µlwasaddedinto each of the cognitive peptide-immobilized wells and they were incubated at room temperature for 20 min and removed of the solution. That remaining in each well on the plate was washed with 200µl of distilled water twice.

Measurement in *EOP*

The amount of CatE trapped on the peptide was directly assayed with the fluorogenic substrate MOCAc-Gly-Ser-Pro-Ala-Phe-Leu-Ala-Lys(Dnp)-D-Arg-NH2(KYS-1) as previously described [27]. Briefly, 5µl of 100µM substrate and 45µl of 50 mM sodium acetate, 100 mM NaCl, pH 4.5, were added to each well and then incubated at 40°C for 10 min. The increase in fluorescence intensity produced by substrate cleavage during incubation was measured at an emission wavelength at 430 nm with excitation at 360 nm using a fluorescence plate reader FluPOLO (TaKaRa, Japan).

Peptide-based sandwich ELISA-like assay (*Sandwich pep-ELISA)*

The other type of peptide apatamer-based assay, CatE trapped onto the microtiter plates by the first cognitive peptide were sandwiched by adding 50µl of the peroxidase-labeled second cognitive peptide aptamer (0.5ng/µl). After incubation at room temperature for 20 min, each well was washed three times with 200µl of distilled water. Then, 100µl of the peroxidase substrate (Quanta Blu Fluorogenic Peroxidase Substrate (TaKaRa, Japan)) was added to each well and incubated at 37°C for 10 min. The fluorescence intensity was measured as described above.

Results and Discussion

A novel technology to identify protease-inhibitory or activating peptides has been developed recently [11]. Owing to this technology, we can easily obtain such peptide aptamers as strongly binding to proteases instead of antibodies. Since peptides can be chemically synthesized in a large quantity, they are apromising substitution for antibodies in various purposes (Immunostaining, Western blotting, Immunoprecipitation and others), especially in ELISA technology.

Analogously to the antibody technology, we can think of two modes of *pep-ELISA*: 'Ensyme-on-peptide (*EOP*)'andpeptide-based Sandwich ELISA-like (Figure 1). Evidently, the former is effective when the binding site of a peptide is apart from its catalytic site and

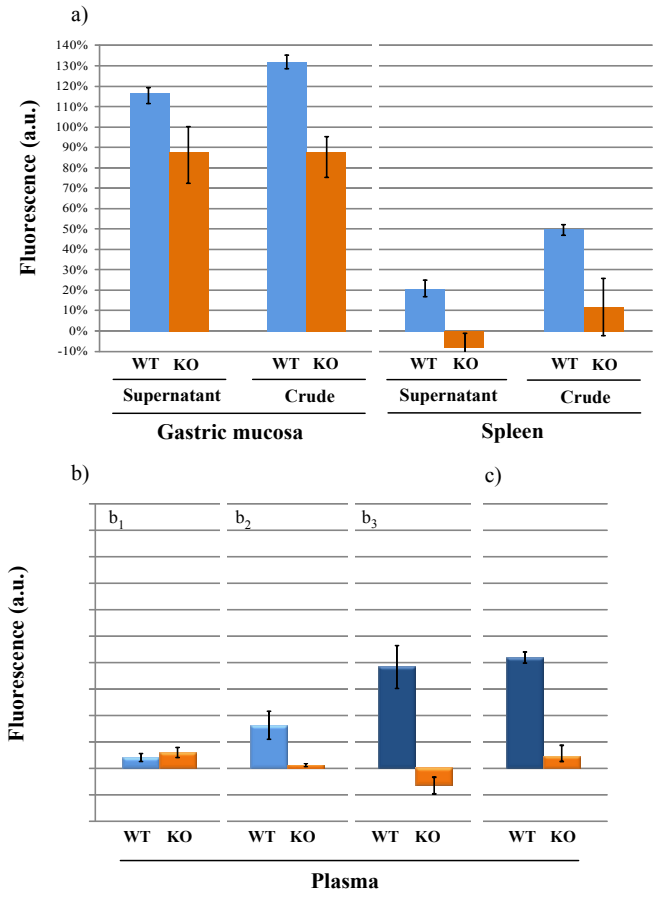

Figure 2: Applications of *EOP* (enzyme-on-peptide)-mode *pep-ELISA*. In this experiment, the fluorescence intensity can be regarded to be proportional to the amount of the enzyme CatE since it generates the fluorescent substrate by cleaving the fluorogenic substrate. a) Measurement of CatE levels of different specimens (rat spleen and gastric mucosa extracted from cathepsin E-gene knockout and wild rats). Since the peptide used (pp2) has a binding (and inhibitory) activity at pH 4.5, these specimens in physiological conditions (at a neutral pH) were pre-treated to adjust the pH to pH 4.5. The aggregates generated in this process were removed (supernatant) or not removed (crude) during the enzyme activity assay. b) The effect of the change in the peptide aptamer composing EOP on the CatE-binding. The peptides used are (b1) non-CatE-binding octapeptide (the sequence of PCSELINT), (b2) pp2 (CatE-inhibitor at pH4.5), and (b3) pp1015 (CatE-activator at pH4.5). The specimens used were plasma extracted from wild type (WT) and knockout (KO) rats. c) CatE measurement by the *EOP-mode pep-ELISA* made of a peptide aptamer (pa4043) selected at a neutral pH (pH7.4). In this case, the trapping of CatE was performed at a neutral pH, where the peptide pa4043is active, while the enzyme activity was monitored at pH 4.5using the supernatant of centrifugation-treated plasma solution(the same origin as in b)which had been subjected topH-alteration from a physiological pH to pH4.5(see Materials and Methods). Note that CatE does not need to be bound to the peptide when its activity is monitored at pH4.5 (where the peptide selected at pH7.4 will probably lose its binding affinity to CatE). Throughout the above experiments, the CatE activity at pH 4.5 was compared, making the comparison of activity easy. Statistics were taken from three independent experiments except b1 (n=2).

the binding of the peptide does not severely impinge on the catalytic activity, which is usually the case with weak inhibitory peptides and activity-enhancing peptides. This type of the peptide aptamer-based ELISA-like assay (briefly, *pep-ELISA*) was constructed using i) a weak inhibitory peptide (pp2 in Table 1) and ii) activating peptides (pp1015 and pa 4043 in Table 1).

Cathepsin E specimens from different tissues (gastric mucosa and spleen) were tested with/without removing the aggregates generated by the pH alteration from the neutral to the acidic (pH4.5). As can be seen in Figure 2a, CatE from both sources can be measured with a difference between the wild (WT) and the knock-out (KO), although the values for the gastric mucosa of the knock-out rats were significantly higher, probably reflecting the abundant existence of family proteases such as cathepsin D in the tissue. Nevertheless, there was a reasonable difference in CatE activity between the wild and the knock-out specimens (Figure 2a). Evidently, there is only a minor difference between with and without removal of the aggregates, indicating the intactness of CatE and the negligible effect of co-precipitation. Since the level of CatE in the wild rat plasma can be estimated to be ~10μg/ml (Supplementary Figure 1), this result shows the sufficient sensitivity of *pep-ELISA*, and is also supported by the fact that this peptide (pp2) hasa sufficiently strong dissociation constant of 570 pM by the surface plasma resonance (SPR) method using a Biacore 2000 (GE Healthcare, UK) in the mode of making a peptide as the ligand and the CatE molecule as the analyte (Table 1).

For the sake of methodological convenience, a CatE-activating peptide in the neutral pH (pa 4043) was selected and used for the same *pep-ELISA* purpose. As can be seen in Figure 2c, this case could also work, meaning the possibility of the direct measurement of blood samples without pH adjustment. The *EOP* result obtained with the neutral pH CatE–activating peptide seems to be supportive of an idea that protease-activating peptides may provide higher sensitivities than inhibitory ones (Figure 2b1& b2) since both peptides, inhibitory and activating, have a comparable Kd (to be published elsewhere).

The peptide-based Sandwich ELISA-like assay (*Sandwich pep-ELISA*) was also tested and shown to be effective (Figure 3, Supplementary Figure 2). So far as we know, this is the first case in which peptides have been shown to be used as substitutes of antibodies. Since the identification of a specific peptide aptamer against a target is very easy in contrast to finding an antibody, we believe the introduced method as a strong impact on the fields of biomolecular sensing and imaging applied to science and medicine.

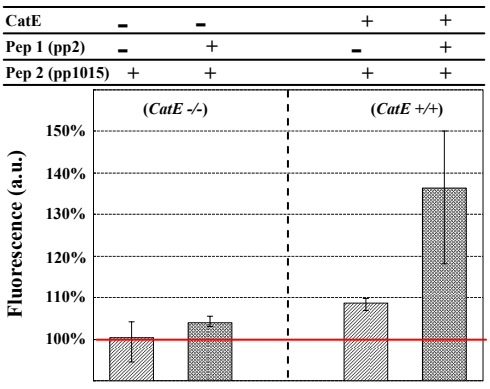

Figure 3: Peptide-based Sandwich ELISA-like assay (*Sandwich pep-ELISA*). In this experiment, the complete sandwich construct (pp2 + CatE + pp1015) and incomplete sandwich construct (CatE + pp1015 without pp2) were tested against the wild and the CatE-gene knockout rat plasma samples. The primary peptidepep 1 (pp2) was immobilized on to the surface of the microplate and then CatE was bound to pep 1 or not. Next, the secondary peptide pep 2 (pp1015) combined with an enzyme horse-radish peroxidase was added, bound to CatE, and then washed to remove the unfixed peroxidase. Note that in this case, CatE works as only a bridge between two peptides and does not work as an enzyme. Statistics were taken from 3 trials.

Inconclusion, CatE-peptide-aptamers could be used for substitutes of antibodies in constructing a peptide-based ELISA-like assay (*pep-ELISA*) for the detection of CatE in tissues and plasma. The modes of *pep-ELISA, EOP* (enzyme-on-peptide) and *Sandwich pep-ELISA,* were all shown to be effective.

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