Preparation of Luciferase-fused Peptides for Immunoassay of Amyloid Beta

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Abstract

An immunoassay, such as the enzyme-linked immunosorbent assay (ELISA), is an analytical method that utilizes the interaction of antigens and antibodies. Enzyme-labeled antigens require both molecular recognition by the antibody and enzymatic activity as a reporter. We designed and constructed an immunodetection system for amyloid beta peptides (Aβ) using an enzyme-labeled antigen expressed from Escherichia coli. Aβ(1–16) fused with renilla luciferase was prepared as the enzyme-labeled antigen. In the presence of this luciferase-fused peptide, the luminescence of coelenterazine-h was observed. The influence of the fusion with Aβ on the luminescence reaction was insignificant. Surface plasmon resonance analysis indicated that the interaction between the luciferase-fused Aβ and anti-Aβ antibody was sufficiently strong. In the competitive ELISA assay for Aβ detection using the luciferase-fused Aβ, the luminescence intensity decreased as the Aβ concentration increased.

Keywords: Amyloid beta, Enzyme-labeled antigen, Fusion protein, Luciferase
Introduction

The enzyme-linked immunosorbent assay (ELISA) is a quantitative analytical method that is based on antigen–antibody interactions. The method relies on sensitivity to antibody recognition, which permits the detection of small quantities of antigens. In the assay, an antigen or antibody is labeled using an enzyme such as alkaline phosphatase (AP) or horseradish peroxidase (HRP); then, a color change in the substrate due to the reaction of the enzyme is detected, enabling quantification of the concentration of an analyte in an unknown sample.

Various types of ELISA that exploit differences in detection procedures have been developed. In particular, sandwich ELISA, which uses two types of antibodies for analyte detection, is known to show remarkably high specificity and sensitivity. Since each antibody is necessary to recognize different epitopes, the selection of suitable antibodies is critical.

Another variant, competitive ELISA, can detect analytes with one type of antibody. This method requires an enzyme-labeled antigen other than a specific antibody. The labeled antigen is employed to compete with the target antigen against the immobilized antibody. Competitive ELISA is an economical method because it requires fewer steps for analysis and uses only one type of antibody compared to sandwich ELISA. However, modification of the antigen with the enzyme may inhibit the antigen–antibody reaction, which may significantly affect ELISA quantification. The detection sensitivity of ELISA depends on the antibody/antigen binding ability and selectivity. In addition, the activity of the enzyme label attached to the antigen used as the reporter molecule and the selection of the substrate are significant factors that affect detection sensitivity. Therefore, the preparation of enzyme-labeled antigens used in competitive ELISA must not compromise either molecular recognition by the antibody or enzyme activity as the reporter.

As mentioned above, the enzymes HRP and AP are widely used as reporter molecules. In particular, the combination of HRP and a chemiluminescent substrate can provide high detection
sensitivity, enabling quantitative analysis of an analyte at low concentration. Conjugation of HRP to antibodies or antigens is performed by organic chemical methods using condensation reagents rather than genetic engineering methods. Use of the former technique often causes a decrease in the interaction ability of the antibody because of the unfavorable modification of epitopes and difficulty in preparing conjugates with a uniform structure. Gene engineering is not generally used for the preparation of a fused protein with a uniform structure, primarily because of the difficulty in the expression of HRP by a protein expression system using host cells such as \textit{Escherichia coli}. Recently, immunoassays using luciferase, a luminescent protein, have attracted research interest. Luciferase exhibits a signal with a high signal-to-noise ratio compared to fluorescent proteins. Owing to the improvement of substrates and luciferase, luciferase has been used in various assays including immunoassays and antibody selection. Since most luciferases can be prepared via the \textit{E. coli} protein expression system, the problems associated with HRP labeling can be overcome. In fact, Ren et al. reported that a luciferase-fused antibody expressed from \textit{E. coli} functioned as a reporter molecule in competitive ELISA. Here, we report our efforts toward the development of a luciferase-labeled antigen produced from \textit{E. coli} and its application in a competitive ELISA system. Our detection target was the peptide, amyloid beta (A\(\beta\)), a known pathogenic molecule for Alzheimer’s disease (AD). Quantitative analysis of A\(\beta\) is an important index for AD diagnosis. In this study, we prepared a \textit{renilla} luciferase (rLuc)-fused A\(\beta\) peptide oriented for the immunodetection of A\(\beta\)(1–40) as an enzyme-labeled antigen. As the prepared antigen indicated good affinity to the anti-A\(\beta\) antibody and sufficient luciferase activity, the rLuc-fused A\(\beta\) was equipped with the functions required as a reporter molecule. A decrease in luminescence intensity was observed in an A\(\beta\) dose-dependent manner when a competitive ELISA system for A\(\beta\)(1–40) was constructed, suggesting the feasibility of the immunodetection of A\(\beta\) using rLuc-A\(\beta\).
**Experimental**

*Preparation of Aβ(1–16) fused with renilla luciferase*

Aβ(1–16) fused with *renilla* luciferase (rLuc-Aβ) was produced by an *E. coli* expression system. To prepare the expression vector, the following three polymerase chain reactions (PCRs) were conducted. First, a DNA template which encoded rLuc with a linker was prepared from the rLuc gene (Addgene #45642: pcDNA3 RLUC POLIRES FLUC<sup>25</sup>) using a standard PCR. The following forward and reverse primers were used for PCR: tttgagctcatgacttcgaaagttttatgat as the forward primer for linker fusion to rLuc and actccctccgccaccactccctcggacttttttgagaacctge as a reverse primer for linker fusion to rLuc. All primers and oligonucleotides were obtained from Thermo Fisher Scientific.

In addition, the PCR product was purified using a DNA cleanup kit (New England Biolabs) and used as a template for the following PCR: tttgagctcatgacttcgaaagttttatgat as a forward primer for Aβ(1–10) insertion and atatcctgagtcatgtcgg as a reverse primer for Aβ(1–10) insertion.

The PCR product was purified using a DNA cleanup kit and used as a template for the following PCR: tttgagctcatgacttcgaaagttttatgat as a forward primer for Aβ(11–16) insertion and tttttgttattttgtgatgagtcgatgtcgg as a reverse primer for Aβ(11–16) insertion.

From the above three PCRs, DNA-encoded rLuc-Aβ was obtained. The prepared linear DNA cleaved by SacI and HindIII was inserted into pColdI (Takarabio). The obtained plasmid was transformed into BL21 cells, and the recombinant protein was expressed and purified using Ni-NTA agarose (Wako Pure Chemical Industries) according to the manufacturer’s instructions. The buffer was exchanged with standard phosphate-buffered saline (PBS) using an NAP-5 column.
(GE Healthcare). The purity of the expressed proteins was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and staining with Coomassie Brilliant Blue R-250 (Wako).

**Dot-blot analysis of rLuc-Aβ using Aβ antibody**

Dot blotting was performed by spotting the Aβ sample directly on a nitrocellulose membrane (Bio-Rad). For immunodetection, membranes were blocked overnight at 4°C with 5% skimmed milk (Wako) in phosphate-buffered saline with Tween 20 (PBST). After blocking, membranes were incubated with mouse monoclonal Aβ antibody 6E10 (BioLegend, 1:2,000) for 1 h at room temperature and then with secondary horseradish peroxidase-conjugated anti-mouse IgG (Abcam, 1:10,000). Proteins were visualized using the Luminata Forte Western HRP substrate (Millipore) according to the manufacturer’s instructions.

**Bioluminescence measurement of coelenterazine using rLuc**

Into PBS solution containing 20 nM rLuc, 10 µM coelenterazine-h (Wako) was added. The mixture was moved to a quartz cell (optical path length, 1.0 cm) and the luminescence spectrum of coelenterazine-h was measured using an LS-55 spectrofluorometer (Perkin Elmer). To assess its biophysical properties, coelenterazine-h at the concentration of 0-15 µM was mixed with 20 nM rLuc in a 96-well microplate. The luminescence of the sample was measured using a microplate reader (Appliskan, Thermo Fisher Scientific). The activity of rLuc was evaluated using the Michaelis-Menten equation (Eq. (1)), where $K_m$ and $V_{max}$ indicate the affinity between the enzyme and substrate and the maximal velocity of the reaction, respectively. These constants were estimated via a nonlinear fitting of the equation using KaleidaGraph 4.0 (Synergy software).

\[
v = \frac{V_{max}[S]}{K_m+[S]} \quad (1)
\]

The parameters $v$ and $[S]$ are the velocity of the reaction and substrate concentration, respectively.
Affinity measurements using surface plasmon resonance

Affinity measurements by surface plasmon resonance (SPR) were performed in HBS-P buffer (10 mM HEPES, pH 7.4; 150 mM NaCl; and 0.05% v/v Surfactant P20) with a Biacore T200 system (GE Healthcare) at 25°C. Aβ antibody 6E10 was dissolved in 10 mM sodium acetate buffer (pH 4.5) and immobilized on a CM5 chip (Cytiva) using the standard amine coupling procedure. The response to immobilization was approximately 300 RU. A flow cell without antibody immobilization served as a control for nonspecific binding, and the flow rate was maintained at 10 µL/min during immobilization. To conduct equilibrium binding measurements, various concentrations (5–250 nM) of Aβ samples were injected into each flow cell at 10 µL/min for 5 min. The binding response was recorded as the difference between the signal of the controlled flow cell and the antibody-immobilized flow cells. Rate constants and dissociation constants were calculated using BIAevaluation software. The plots were fitted to 1:1 Langmuir binding model to determine the apparent association ($k_a$) and dissociation rate constants ($k_d$). A affinity between antibody and Aβ samples was described by dissociation constant $K_d$ ($= k_d/k_a$).

Competitive ELISA for the detection of Aβ(1–40) using rLuc-Aβ

Aβ antibody 6E10 was biotinylated using a biotin-labeling kit (Dojindo Molecular Technologies, Inc.), and the biotin-modified antibody was immobilized in a 96-well avidin-coated microplate (Sumitomo Bakelite) in the presence of 1% w/v bovine serum albumin (BSA) dissolved in PBST solution (containing 0.05% w/v Tween 20). Into the microplate well, 100 µL 1 µg/mL Aβ antibody solution was added and incubated for 1 h at room temperature. After incubation, the wells were washed three times with 200 µL PBST solution. Into the antibody-immobilized microwell, sample (100 µL) containing both 1 µM Aβ(1–16) fused with rLuc and various concentrations of Aβ(1-40) (0–10 µM) was poured and then incubated for 1.5 h at 37°C. After incubation, the wells were
washed three times with 200 µL PBST solution. Then, 100 µL of 10 µM coelenterazine-h-containing PBS solution was added, and the bioluminescence of coelenterazine-h was measured using the Appliskan microplate reader.

Results and Discussion

As an enzyme-labeled antigen employed in competitive ELISA for Aβ detection, Aβ(1–16) fused with rLuc was prepared (Fig. 1). After purification of the luciferase-fused protein expressed in E. coli, protein expression was confirmed using SDS-PAGE. Single bands of rLuc (estimated MW 38.4 kDa) and rLuc-Aβ (estimated MW 40.1 kDa) are observed, as shown in Fig. 2a. The prepared luciferase-fused protein (rLuc-Aβ) is capable of capturing the antiamyloid beta antibody 6E10 because Aβ(1–16) is an epitope sequence of 6E10. To confirm the interaction of the prepared luciferase-fused protein with 6E10, a dot-blot analysis was performed. Spots of Aβ(1–40), rLuc, and rLuc-Aβ immobilized on a nitrocellulose membrane were treated with both 6E10 and HRP-conjugated anti-IgG antibodies and visualized by chemiluminescence. Although the spot of rLuc that did not possess an epitope sequence of 6E10 was undetected, bright signals were confirmed in the spots of Aβ(1–40) and rLuc-Aβ (Fig. 2b). The results indicate that the prepared rLuc-Aβ has the ability to interact with the Aβ antibody, as expected.

Next, we assessed the luciferase activity of rLuc-Aβ. Figure 3a shows the luminescence spectrum of coelenterazine-h that was employed as a substrate for the rLuc activity assay. The spectrum in the presence of rLuc-Aβ exhibits a typical peak at approximately 480 nm. Although the luminescence intensity at 480 nm of the sample treated with rLuc-Aβ decreased slightly compared to that of rLuc, the influence of luciferase fusion on the luciferase activity of rLuc-Aβ was limited. The biophysical constants for rLuc based on the Michaelis-Menten equation were obtained from plots of the initial velocity of the enzymatic reaction vs. substrate concentration (Fig. 3b). The parameters calculated from the curve fitting are indicated in Table 1.

Although the $K_m$ value of
rLuc-Aβ is about twice as large as that of rLuc. Aβ fusion has little influence on the luminescence intensity of rLuc. Thus, rLuc-Aβ can be used for competitive ELISA as a reporter molecule because sufficient luminescence intensity is observed.

The dissociation constant for the interaction between rLuc-Aβ and anti-Aβ antibody influences the detection sensitivity of competitive ELISA. SPR analysis of the protein–protein interaction using the Biacore system was carried out to assess the recognition ability of the anti-Aβ antibody toward rLuc-Aβ. Several concentrations of Aβ samples were injected into the flow cell, and then their interactions with 6E10 immobilized on a sensor chip were measured. Figure S1 shows the sensorgrams that reflect the interaction of 6E10 with each Aβ sample. Both sensorgrams show a concentration-dependent increase in response to intensity. The sensorgrams were fitted with a 1:1 binding fitting model using the BIAevaluation program. The obtained values of the kinetic parameters are shown in Table 2. The dissociation constant $K_d$ of rLuc-Aβ is nearly twice that of Aβ. Although the dissociation rate constants $k_d$ of both rLuc-Aβ and Aβ samples are comparable, the association rate constant $k_a$ of rLuc-Aβ is smaller than that of Aβ. These results indicate that the fusion of rLuc with Aβ slightly inhibits the association of Aβ with 6E10. However, the interaction between the luciferase-fused protein and 6E10 is sufficiently strong since the dissociation constant is approximately 70 μM. Thus, we considered that rLuc-Aβ is available for competitive ELISA as an enzyme-labeled antigen.

Finally, the competitive ELISA assay for Aβ detection using rLuc-Aβ was performed. Figure 4 shows the relationship between the concentration of Aβ(1–40) and the luminescence intensity of coelenterazine-h. The luminescence intensity decreases substantially as the Aβ concentration increases, suggesting that the immunodetection of Aβ(1-40) using rLuc-Aβ occurs as expected. On the other hand, the measurement error increases depending on the Aβ concentration, and it is not possible to obtain an accuracy profile consistent with the theory of competitive ELISA. It is thought that the short luminescence half-life of coelenterazine-h greatly affected the random error,
which prevented us from obtaining the accuracy required for quantitative analysis. Therefore, further investigation of the luciferase and substrate combination is necessary to construct a quantitative analytical method.

**Conclusions**

In this study, a system for Aβ detection was constructed by fusing Aβ(1-40) with rLuc using a protein engineering method and evaluated by applying the luciferase-fused protein in competitive ELISA. Although the luciferase-fused protein effectively functioned as a reporter molecule for immunodetection, a better combination of enzyme and substrate that minimizes measurement error is required to construct a quantitative assay system. Since Aβ antibody 6E10 binds not only to Aβ(1–40) but also to other Aβ peptides, including Aβ(1–16), the rLuc-fused Ab could be used to detect most of the Aβ present in the brain. By using an antibody specific to the amino acid length of Aβ, construction of an Aβ-length-selective immunodetection system should be accessible by employing a procedure similar to that used in the present study.

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### Table 1  Substrate influence on the kinetic parameters of luciferase

| rLuc | rLuc-Aβ |
|------|---------|
| $V_{\text{max}}/\text{RLU} \cdot \text{s}^{-1}$ | $K_m/\mu\text{M}$ |
| $6.9 \times 10^7$ | 0.7 |
| $6.5 \times 10^7$ | 1.7 |

### Table 2  Dissociation constants between Aβ and antibody 6E10

| Aβ     | $k_a \times 10^4/\text{M}^{-1} \cdot \text{s}^{-1}$ | $k_d/\text{s}^{-1}$ | $K_d/\mu\text{M}$ |
|--------|---------------------------------|-----------------|-----------------|
|        | $k_a \times 10^4/\text{M}^{-1} \cdot \text{s}^{-1}$ | $k_d/\text{s}^{-1}$ | $K_d/\mu\text{M}$ |
| Aβ     | 4.38                            | 1.48            | 33.8            |
| rLuc-Aβ | 1.65                            | 1.20            | 73.0            |
Figure Captions

**Figure 1** Illustration of fusion between Aβ(1–16) and luciferase. The N terminal of Aβ(1–16) was linked to the C terminal of rLuc via a linker sequence.

**Figure 2** Synthesis of Aβ(1–16) fused with luciferase. (A) SDS–PAGE profiles of expressed proteins using 10–15% gradient gel. rLuc and rLuc-Aβ indicate luciferase and Aβ(1–16) fused with luciferase, respectively. (B) Dot-blot analysis using anti-Aβ antibody 6E10. Dot-blotting was performed by spotting the Aβ sample directly on a nitrocellulose membrane. After blocking, the membranes were incubated with mouse monoclonal Aβ antibody 6E10 for 1 h at room temperature and then with secondary horseradish peroxidase-conjugated anti-mouse IgG. Proteins were visualized using a chemiluminescent substrate.

**Figure 3** Assessment of enzymatic activity of luciferase using coelenterazine-h as a substrate. (A) Luminescence spectrum derived from coelenterazine-h. Lines a and b indicate the presence of rLuc and rLuc-Aβ, respectively. (B) Effect of coelenterazine-h substrate concentration on luciferase activity. A nonlinear fitting of the Michaelis–Menten equation was carried out using KaleidaGraph 4.0.

**Figure 4** Plots of luminescent intensity against Aβ concentration. \(I/I_0\) indicates the ratio of luminescence intensity in the presence of Aβ(1–40) to that in the absence of Aβ. Biotin-modified antibody 6E10 was immobilized in a 96 well avidin-coated microplate in the presence of 1% w/v BSA dissolved in PBST solution. A sample containing both 1 μM rLuc-Aβ and various concentrations of Aβ(1-40) (0–10 μM) was poured into the antibody-immobilized microwell and then incubated for 1.5 h at 37°C. Coelenterazine-h-containing PBS solution was added into the
microwell, and then the bioluminescence of coelenterazine-h was measured. Each test was replicated 3 times.

**Figure S1** SPR sensorgrams of Aβ sample binding to the immobilized antibody 6E10 on a CM5 sensor chip. Various concentrations (5–250 nM) of Aβ were injected for 5 min at a flow rate of 10 µL/min. The arrows indicate the increase of Aβ concentrations from 5–250 nM in the sensorgrams.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. S1
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