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Original Research Paper

Biochemistry

ENHANCEMENT OF THE SENSITIVITY OF SOLUBLE AMYLOID BETA PROTEIN1-42 AND VALIDATION OF ITS DETECTION IN A MIXED PROTEIN SOLUTION

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ABSTRACT Aβ aggregation occurs preferentially and accumulates in the brains of patients with Alzheimer's disease. Previously, we developed monoclonal antibodies against aggregated $Aβ_{1-42}$ and soluble $Aβ_{1-42}$, enabling us to distinguish aggregates with different structures by utilizing these antibodies. To apply the monoclonal antibodies against aggregated $Aβ_{1-42}$ for clinical diagnosis, detecting trace amounts of amyloid in the blood is essential, thus necessitating a test method with high detection sensitivity and specificity. We evaluated an assay system for detecting soluble $Aβ_{1-42}$aggregates in protein preparations and identifying them in serum samples. We focused on blood detection and identified soluble aggregates of $Aβ_{1-42}$in a protein mixture solution using the antibodies we developed, evaluating them through enzyme-linked immunosorbent assays (ELISA). We also conducted ELISA using a chemiluminescent substrate and explored methods to enhance detection sensitivity and reaction specificity. Consequently, highly sensitive measurement results were achieved even in a protein mixture solution. Furthermore, detection using a chemiluminescent substrate yielded a detection sensitivity approximately five times higher than that of the conventional color substrate we previously reported. These methods are potentially applicable to clinical diagnosis as specific detection methods in blood.

KEYWORDS : Alzheimer's Disease, Amyloid Beta $Protein_{1-42}$, Enzyme-Linked Immunosorbent Assays, Monoclonal Antibodies, Chemiluminescent Substrates, Detection Sensitivity, Reaction Specificity

INTRODUCTION

Amyloid beta protein (Aβ) is synthesized in various brain cells through the cleavage of its precursor, amyloid precursor protein, by β-secretase and γ-secretase (Kang et al., 1987; Matsui et al., 2007). Aβ is generally categorized into forms with 40 amino acid residues and those with 41 amino acid residues, based on the secretase involved. $Aβ_{1-42}$, despite its low purification yield, is highly prone to aggregation and has been reported to exhibit significant neurotoxicity (Borchelt et al., 1996; Kimberly et al., 2003; Suzuki et al., 1994). It is established that $Aβ_{1-42}$ aggregates in the brains of individuals with Alzheimer's disease, although the underlying reasons remain unclear (Andreasen & Blennow, 2002; Galzitskaya, 2019; Gurry & Stultz, 2014). Numerous studies have investigated the aggregation of the Aβ protein, categorizing these aggregates into fibrillar and oligomeric types characterized by beta sheet structures (Matsumura et al., 2011). However, the mechanisms and morphologies involved in the aggregation process exhibit considerable variability (Hosoi et al., 2023; Quan et al., 2023; Wu et al., 2021). In our previous work, we successfully produced $Aβ_{1-42}$ aggregates and developed a flowchart delineating the aggregation pathway (Shimizu et al., 2013a). Two primary pathways have been identified in the aggregation process of $Aβ_{1-42}$. The first pathway involves a transition from monomer to dimer, then to a spherical structure, and ultimately to a fibril structure. The second pathway involves the aggregation of monomers into spherical aggregates of varying sizes, including oligomer-like and even large spherical aggregates. We have also generated monoclonal antibodies targeting both aggregate and soluble forms of $Aβ_{1-42}$ and conducted comprehensive analyses of their reactivity and specificity (Shimizu et al., 2013b; Shimizu et al., 2015). However, to utilize these monoclonal antibodies for the clinical diagnosis of Alzheimer's disease, it is imperative to detect trace amounts of amyloid in the blood, thus necessitating a test method with high sensitivity and specificity.

Chemiluminescent substrates are frequently employed to enhance the detection sensitivity of enzyme-linked immunosorbent assays (ELISA). The choice of substrate is contingent upon the specific enzyme conjugated to the secondary antibody, with horseradish peroxidase (HRP) being the most utilized enzyme, which can be paired with various compounds. In this study, the SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific) was utilized, which is reported to detect protein antigens at a concentration of 0.17 pg/well and a measurement wavelength of 425 nm.

In the sandwich ELISA technique, an antibody is initially immobilized on a solid-phase support, such as a plate. Subsequently, an antigen binds to this antibody, followed by the reaction of an enzyme-labeled antibody against the antigen, serving as a secondary antibody. An enzyme substrate solution is then introduced to induce color or light development, and the reactivity is quantitatively assessed using a microplate reader. The selection of different absorption or emission wavelengths is contingent upon the type of enzyme and substrate solution employed to label the secondary antibody. When serum or other samples are utilized as specimens, the primary antibody selectively captures the target protein from a mixture of numerous proteins, anchoring it to the plate. This process concentrates the target protein antigen, thereby enhancing the detection sensitivity of the enzyme antibody method using the secondary antibody. Several studies have reported the detection of $Aβ_{1-42}$ using sandwich ELISA for clinical diagnosis. A previous study reported the measurement of Aβ in the plasma of patients with Alzheimer's disease using sandwich ELISA with monoclonal antibody BNT77 against Aβ11-28, as well as monoclonal antibodies BA27 and BC05 against $Aβ_{1-40}$ and $Aβ_{1-42}$, respectively (Andreasen & Blennow, 2002). Furthermore, efforts have been made to assess the risk of developing Alzheimer's disease by measuring the ratio of $Aβ_{1-}$

$_{40}$ to $A\beta_{1-42}$ concentrations in plasma using a sandwich ELISA with the 6E10 monoclonal antibody (Cotton et al., 1973). These findings suggest the presence of monomeric or aggregated $A\beta_{1-42}$ in the blood of patients with Alzheimer's disease; detecting the state of Aβ in the blood is considered instrumental in diagnosing the disease. In this study, we investigated strategies to improve the detection sensitivity and reaction specificity of the previously mentioned chemiluminescent ELISA, employing the monoclonal antibodies we have developed. Furthermore, we evaluated an assay system for detecting soluble $A\beta_{1-42}$ aggregates in protein preparations and identifying them in serum samples.

MATERIALS AND METHODS

Comparison of Color- and Luminescence-based Sandwich Methods

In the colorimetric assay, 100 μL/well of monoclonal antibody 73-1, targeting soluble aggregates of $A\beta_{1-42}$ at a concentration of 2 μg/mL, was introduced as the primary antibody into a 96-well plate; the plate was incubated at 37 °C for 1 h to facilitate antibody binding to the plate. Following aspiration to remove the antigen, the plate was washed five times with 200 μL/well of phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBS-T). Subsequently, PBS-T was removed, and 200 μL/well of blocking buffer (Immunoblock, DS Pharma Biomedical) was added; the plate was incubated at 37 °C for 2 h. The plate underwent another five washes with PBS-T at 200 μL/well, after which 0–22 pmol/well of soluble aggregates of $A\beta_{1-42}$ (0.22 M) was added as the antigen. Following a 1-h reaction at 37 °C, the antigen solution was removed, and the plate was washed five times with 200 μL/well of PBS-T.

The secondary antibody, 100 μL/well of HRP-labeled monoclonal antibody 79-3 clone at a concentration of 0.144 μg/mL, was then added. After a 1-h reaction at 37 °C, the plate was washed five times with 200 μL/well of PBS-T. A substrate solution was prepared by combining 10 mg of O-phenylalanine dichloride (OPD) (Sigma-Aldrich, St. Louis, MO) and 5 μL of hydrogen peroxide with 25 mL of citrate buffer. Subsequently, 100 μL/well of this solution was added and reacted at 25 °C for 20 min in the dark.

To terminate the enzyme reaction, 50 μL/well of 0.5 M sulfuric acid was added. Absorbance was measured at a wavelength of 492 nm using a microplate reader. In the luminescence assay, 100 μL/well of monoclonal antibody 73-1 against soluble aggregates of $A\beta_{1-42}$ was added to a 96-well plate as the primary antibody at a concentration of 2 μg/mL; the plate was incubated at 37 °C for 1 h to facilitate antibody binding to the plate. The antigen was removed by aspiration, and the plate was washed five times with 200 μL/well of PBS-T. Subsequently, PBS-T was removed, and 200 μL/well of blocking buffer (Immunoblock, DS Pharma Biomedical) was added; the plate was incubated at 37 °C for 2 h. The plate was again washed five times with PBS with 0.1% Tween at 200 μL/well, and 0.22 pmol/well of soluble aggregates of $A\beta_{1-42}$ (0.22 μM) was added as the antigen. Following a 1-h reaction at 37 °C, the antigen solution was removed, and the plate was washed five times with 200 μL/well of PBS-T. The secondary antibody, 100 μL/well of HRP-labeled monoclonal antibody 79-3 clone at a concentration of 0.144 μg/mL, was added. After a 1-h reaction at 37 °C, the plate was washed five times with 200 μL/well of PBS-T. Subsequently, 100 μL/well of SuperSignal ELISA Femto Maximum Sensitivity Substrate, a chemiluminescent substrate, was added and reacted at 25 ℃ for 1 min. Luminescence intensity was measured at a wavelength of 425 nm using a microplate reader.

Comparison of the Sandwich and Direct Methods in Protein Mixtures

To examine the detection of soluble aggregates of $A\beta_{1-42}$ in conjunction with other proteins, a sandwich ELISA (73-1 and 79-3) was conducted using a mixture of soluble aggregates of $A\beta_{1-42}$ and bovine serum albumin as the antigen. Monoclonal antibody 73-1, targeting soluble $A\beta_{1-42}$ at a concentration of 2 μg/mL, was employed as the primary antibody. It was added to a 96-well plate at 100 μL/well; the plate was incubated at 37 °C for 1 h to facilitate antibody binding to the plate. Following aspiration to remove the antigen, the plate was washed five times with 200 μL/well of PBS-T. Subsequently, PBS-T was removed, and 200 μL/well of blocking buffer (Immunoblock, DS Pharma Biomedical) was added; the plate was incubated at 37 °C for 2 h. The plate was then washed three times with PBS-T at 200 μL/well, and soluble aggregates of $A\beta_{1-42}$ albumin were introduced to each well of the 96-well plate as the antigen (0.22 pmol/well, adjusted to a total protein concentration of 50 ng/mL at ratios of 1:0, 1:1, 1:5, 1:10, and 0:1).

After a 1-h reaction at 37 °C, the antigen solution was removed, and the plate was washed five times with 200 μL/well of PBS-T. The secondary antibody, HRP-labeled monoclonal antibody 79-3 clone at a concentration of 0.144 μg/mL, was added at 100 μL/well. Following a 1-h reaction at 37 °C, the plate was washed five times with 200 μL/well of PBS-T. A substrate solution was prepared by adding 10 mg of OPD and 5 μL of hydrogen peroxide to 25 mL of citrate buffer. This solution was added at 100 μL/well and reacted at 25 °C for 20 min in the dark. To terminate the enzyme reaction, 50 μL/well of 0.5 M sulfuric acid was added.

The absorbance of each well was subsequently measured at 492 nm using a microplate reader. For the direct ELISA method, the albumin mixture was prepared as an antigen and added at 0.22 pmol/well. After a 1-h reaction at 37 °C, the antigen was removed by aspiration, and the plate was washed five times with 200 μL/well PBS-T. PBS-T was then removed, and blocking buffer (Immunoblock, DS Pharma Biomedical) was added at 200 μL/well; the plate was incubated at 37 °C for 2 h.

The plate was washed three times with PBS-T at 200 μL/well, and 100 μL/well of HRP-labeled monoclonal antibody 79-3 clone at a concentration of 0.144 μg/mL was added as the secondary antibody. Following a 1-h reaction at 37 °C, the plate was washed five times with 200 μL/well of PBS-T. A substrate solution was prepared by adding 10 mg of OPD and 5 μL of hydrogen peroxide to 25 mL of citrate buffer. This solution was added at 100 μL/well and reacted at 25 °C for 20 min in the dark. To halt the enzyme reaction, 50 μL/well of 0.5 M sulfuric acid was added. The absorbance of each well was then measured at a wavelength of 492 nm using a microplate reader.

Detection of Soluble Aggregates of $A\beta_{1-42}$ in Protein Mixtures

A chemiluminescence ELISA was conducted to detect trace antigens in serum, utilizing a mixture of soluble aggregates of $A\beta_{1-42}$ and bovine serum albumin as the antigen. The mixture ratio was calibrated to ensure a total protein concentration of 500 ng/mL per well in a 96-well plate, with 100 L added to each well. The specific ratios of Aβ were adjusted to 100% (11 pmol/well), 50% (5.5 pmol/well), 20% (2.2 pmol/well), 10% (1.1 pmol/well), 5% (0.55 pmol/well), 2% (0.22 pmol/well), 1% (0.11 pmol/well), and 0% (0 pmol/well), with the remaining concentration supplemented by albumin solution to achieve a total of 50 ng/well, which was then immobilized on the plate.

As a control, a dilution series of soluble aggregates of $A\beta_{1-42}$ was prepared without albumin and immobilized similarly. The antigen was aspirated, followed by the addition of 200 μL/well of PBS-T for washing. Subsequently, the PBS-T was removed, and 200 μL/well of blocking buffer (Immunoblock, DS Pharma Biomedical) was added; the plate was incubated at 37 °C for 2 h. The plate underwent five washes with PBS-T (200 μL/well), and 100 μL/well of HRP-labeled monoclonal antibody 79-3 clone (0.144 μg/mL concentration) was introduced as the

secondary antibody. After a 1-h reaction at 37 °C, the plate was washed five times with 200 μL/well of PBS-T. Subsequently, 100 μL/well of SuperSignal ELISA Femto Maximum Sensitivity Substrate, a chemiluminescent substrate, was added and reacted at 25 °C for 1 min. The luminescence intensity was then measured at a wavelength of 425 nm using a microplate reader.

RESULTS

Comparison of the Color- and Luminescence-based Sandwich Methods

we conducted a sandwich ELISA to compare the reactivity of the chemiluminescence method with the colorimetric method, while also investigating strategies to enhance sensitivity (Figure 1). The colorimetric method detected concentrations ≥ 11 pmol/well, whereas the chemiluminescence method detected concentrations ≥ 2.2 pmol/well, thereby confirming an approximate fivefold increase in sensitivity.

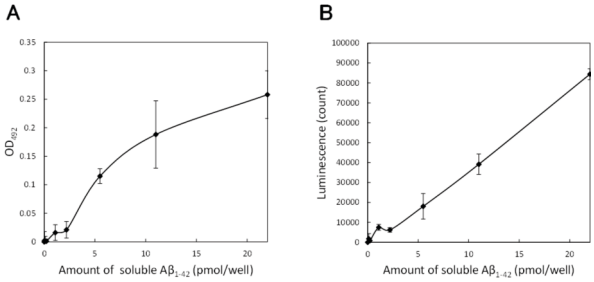

Figure 1. Evaluation of the reactivity of chemiluminescence and absorbance techniques in sandwich ELISA. (A) Sandwich ELISA (colorimetric method, 73-1 and HRP labeled 79-3). (B) Sandwich ELISA (chemiluminescence method, 73-1 and HRP-labeled 79-3). Data are presented as means ± S.D. [n=3]. ELISA, Enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; SD, standard deviation; Aβ, Amyloid beta protein; OD, optical density

Comparison of the Sandwich and Direct Methods in Protein Mixtures

In vivo and within the bloodstream, $Aβ_{1-42}$ aggregates are not isolated entities but are intermingled with other proteins. Initially, a comparison of the color development step of ELISA was made between the sandwich and direct methods, utilizing albumin as the protein mixture. When soluble aggregates of $Aβ_{1-42}$ were used independently, the sensitivity of the sandwich ELISA was approximately 5.8 times greater than that observed with the direct ELISA (Figure 2). Furthermore, when soluble aggregates of $Aβ_{1-42}$ were combined with albumin, the sandwich ELISA could detect up to one-fifth of the total protein content.

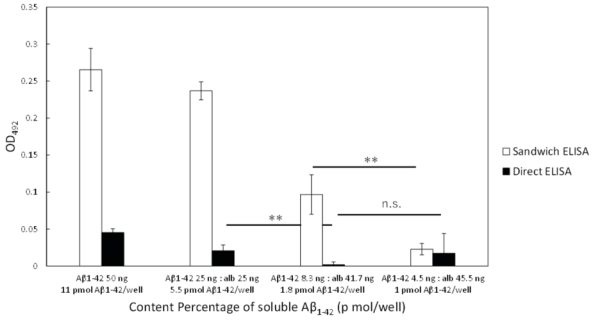

Figure 2. Quantification of solubilized $Aβ_{1-42}$ in albumin mixtures utilizing sandwich ELISA. Evaluation of the reactivity of sandwich ELISA versus direct ELISA across different concentrations of $Aβ_{1-42}$. **p < 0.05 (unpaired two-group Student's t test). Data are presented as means ± S.D. [n=3]. ELISA, Enzyme-linked immunosorbent assay; SD, standard deviation; Aβ, Amyloid beta protein; OD, optical density

Detection of Soluble Aggregates of $Aβ_{1-42}$ in Protein Mixtures by Chemiluminescence ELISA

Building on the findings outlined in the previous section, albumin was employed to detect soluble aggregates of $Aβ_{1-42}$ within the protein mixture via chemiluminescence ELISA. Soluble aggregates of $Aβ_{1-42}$ served as the antigen, with Aβ concentrations adjusted from 100% to 0% (11–0 pmol/well) of the total; the remaining Aβ concentration was supplemented with albumin solution to achieve a total of 50 ng/well, which was then immobilized on the plate. A separate control experiment involved a dilution series of soluble aggregates of $Aβ_{1-42}$ without albumin, immobilized on the plate in a similar manner. The reactivity with HRP-labeled 79-3 monoclonal antibody was assessed under each condition using chemiluminescence ELISA (Figure 3). The findings indicated that Aβ could be detected at 10% of the total, equivalent to ≥ 1.1 pmol/well, regardless of the presence or absence of albumin.

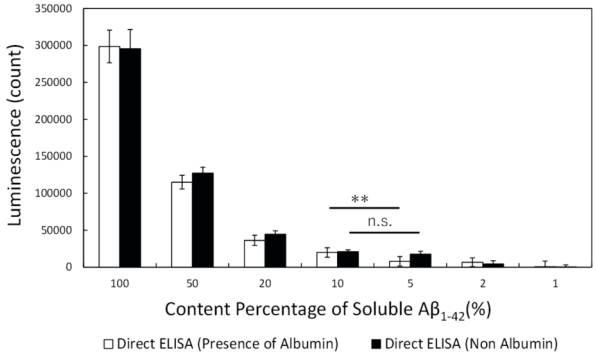

Figure 3. Detection of solubilized $Aβ_{1-42}$ in protein mixtures, conducted using the monoclonal antibody clone 79-3 through chemiluminescence ELISA. Solutions were prepared with varying ratios of albumin and were compared with solutions containing albumin and those lacking albumin. **p < 0.05 (unpaired two-group Student's t-test). Data are presented as means ± S.D. [n=3]. ELISA, Enzyme-linked immunosorbent assay; SD, standard deviation; Aβ, Amyloid beta protein.

DISCUSSION

In this study, we validated the detection of soluble aggregates of $Aβ_{1-42}$ within a protein mixture that simulates actual blood measurement and explored a method to enhance the sensitivity and specificity of the antigen-antibody reaction for ELISA diagnosis of $Aβ_{1-42}$ aggregates. Previously, we employed a sandwich ELISA method, utilizing various monoclonal antibodies against soluble aggregates of $Aβ_{1-42}$, to assess the reaction specificity for different aggregated forms of $Aβ_{1-42}$. Our findings indicated that the 79-3 monoclonal antibody exhibited the highest reactivity when paired with the 73-1 monoclonal antibody (Shimizu et al., 2013b). In this study, we continued to use the combination of the 73-1 and 79-3 monoclonal antibodies to improve reactivity and specificity.

In the context of actual blood-based diagnosis, $Aβ_{1-42}$ aggregates do not exist in isolation within serum but are mixed with proteins such as albumin. Therefore, to enhance specificity and sensitivity, we conducted a chemiluminescence ELISA using a mixture of $Aβ_{1-42}$ aggregates and bovine serum albumin as the antigen and compared the reaction sensitivity. Our results demonstrate that the sensitivity of the sandwich ELISA, employing the chemiluminescence method previously described, can be increased to 2.2 pmol/well. Furthermore, soluble aggregates of $Aβ_{1-42}$ were detected at concentrations of ≥ 1.1 pmol/well in a protein mixture similar to serum, irrespective of the presence or absence of albumin. This value aligns with the detection limit for the large oval aggregate of the monoclonal antibody 37-11 against aggregate $Aβ_{1-42}$, as previously reported. However, given that $Aβ_{1-42}$ is mixed with other proteins in serum, it is posited that a more sensitive detection system has been established. These findings also indicate that the antibody and detection system we developed can detect soluble

aggregates of $A\beta_{1\text{-}42}$ at levels of several pmol/well in a protein mixture.

Notably, the concentration of aggregated Aβ in plasma is 624.5 ng/mL, and antibodies against solubilized $A\beta_{1\text{-}42}$ are present at approximately 0.5 μg/mL in the serum of patients with Alzheimer's disease (Klaver et al., 2011; Zhou et al., 2012). This report posits that, in addition to Aβ monomers, $A\beta_{1\text{-}42}$ aggregates measuring 50–100 nm may also be present in the serum of patients with Alzheimer's disease. A distinctive feature of our research is the creation of various aggregates and the mapping of the aggregation reaction by organizing their morphological changes. Additionally, by generating and combining monoclonal antibodies against aggregates of different sizes, it has become feasible to detect aggregates of varying sizes.

It is hypothesized that aggregates of various sizes, including monomers, exist in the body, depending on the progression of Alzheimer's disease. Regarding the aggregation process, the antibody and detection system developed in this study, characterized by high specificity and sensitivity, respectively, are considered valuable tools for understanding the progression of Alzheimer's disease. This model is considered highly effective for detecting $A\beta_{1\text{-}42}$ in the serum. These results are expected to contribute to the practical application of diagnostic drugs for patients with Alzheimer's disease in the future.

CONCLUSION

In this study, we explored methods to enhance the detection sensitivity and specificity of ELISA reactions for soluble aggregates of $A\beta_{1\text{-}42}$, with a focus on clinical applications. We achieved a fivefold increase in detection sensitivity by employing a chemiluminescent substrate. As a detection method with potential for in vivo application, we conducted a chemiluminescent ELISA using a mixture of soluble aggregates of $A\beta_{1\text{-}42}$ and bovine serum albumin as the antigen. Our findings indicate that the same quantity of $A\beta_{1\text{-}42}$ can be detected irrespective of the presence or absence of albumin. These results hold promise for the practical application of in vivo $A\beta_{1\text{-}42}$ detection methods and the development of diagnostic drugs for Alzheimer's disease.

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