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New Methodology for Estimation of the Prion Protein 106-126 Amyloid Aggregation

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Prion disorders manifest when the prion protein (PrP^{C}) undergoes a conformational change from a α-helical structure to a β-sheet rich structure, which is a pathogenic form (PrP^{Sc}) that results in intracellular PrP^{Sc} aggregation and deposition in the brain.[1]

The synthetic peptide, PrP 106-126 (KTNMKHMAGAA AAGAVVGGLG), exhibits fibrillogenicity and toxicity to neurons *in vitro*. It shares many biological properties with PrP^{Sc} including β-sheet rich structure, resistance to protease digestion as well as the activation of astroglial and microglial cells *in vitro*. It also has similar toxicity to cause cell's death with PrP^{Sc}.[2]

Researches on the discovery of drugs against amyloidogenic proteins or oligopeptides which cause amyloidoses, such as AD (Alzheimer disease), type 2 diabetes mellitus, Parkinson's disease, and mad cow disease, have focused on reducing the level of amyloid formation, cleaving amyloidogenic peptides, or blocking aggregation with organic molecules that selectively bind and inhibit aggregation and fibril formation.[3-8]

Thioflavin-T assay, UV-CD spectroscopy assay, and immunoblotting assay are widely used to determine the relative quantity of amyloid in studies of amyloid-preventing drugs.[8-10] These methods have focused on the aggregated fibril morphology not on the quantitative analysis of individual peptide for the detection of amyloids/fibrils. Recent papers have reported that the main cause of amyloid related diseases are not from fibril or plaque but from oligopeptides that are composed small numbers of peptides.[11-13] Those assays are designed to detect only aggregated fibril, therefore cannot distinguish the fibril states; pentamer, hexamer, heptamer, protofibril, fibril, or plaque. It is impossible to determine the quantity of detected fibrils. Unfortunately, there have been no reports on how many monomers compose the detectable fibrils. Further studies are required to understand the relationship between detection and the composition of fibrils.

The method employed in the present study sheds a light for determining the level of PrP 106-126 aggregation. The precise quantity from the monomer to pentamer of PrP 106-206 peptide is measured by using size exclusion filtration. The aggregated peptides of which sizes are larger than 10,000 molecular weight are removed. Then, filtrate containing monomer to pentamer is hydrolyzed and fluorescence analysis by fluorescamine was conducted. The mean fluorescence intensity of 4 experiments was used to determine the peptide quantity. It is easy to operate and quite sensitive to many small sample quantities (100 μL sample is enough to analyze in 96 well plate).

The phenolic yellow curry pigment, curcumin, has potent antioxidant, anti-inflammatory, and potential cancer chemotherapeutic activities.[15] Curcumin is a potent inhibitor of prion protein amyloid formation *in vitro*.[16] In curcumin inhibition studies on the prion protein, the conventional methods including Th-T, UV-CD spectroscopy, and immunoblotting assay methods were employed.

The ability of curcumin to reduce the extent of aggregates of which is larger than pentamer is evaluated by using a novel method developed in previous research.[14] The extent of aggregation of PrP 106-126 after 2 hours of incubation was estimated by quantifying the amount of peptides smaller than hexamer. Then, the estimated quantities were used for reference data. The effectiveness of curcumin to reduce the aggregation of larger oligopeptides was also measured by same approach (Fig. 2).

The rate of PrP 106-126 aggregation of size greater than pentamer was estimated to be 73.7 and 73.5% after 2 hours incubation at 37 °C in rotated reactor (30 RPM) and non-rotated reactor, respectively. This shows that the aggregation rate is not increased by rotation and approximately 26.4% monomers and oligomers (smaller than the hexamer) exist in the reactor after incubation *in vitro*.

Curcumin has been reported to inhibit aggregation, promote the disaggregation of β-amyloid, and bind to the α-helical intermediate of the prion protein at a curcumin to PrP ratio of 1 : 2.[18] However, its rate of inhibiting fibril formation, particularly for the oligomers, has not been determined yet due to the absence of proper method. The estimation of the PrP 106-126 aggregation blocking rate is enabled by using the procedure mentioned above. The rate of PrP 106-126 aggregation for the fibril formation, which is greater than pentamer in a curcumin phosphate buffer solution, was 62.8% after 2 hours incubation in 37 °C at a curcumin to PrP 106-126 ratio of 1 : 1. This result shows that curcumin has an effect to inhibit the formation of PrP 106-126 fibrils over the range larger than the pentamer by 10.9% *in vitro*.

Experimental

1 mg of PrP 106-126 (Peptron, more than 95% purity level powder) was dissolved in 1 mL 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP, purchased from Sigma) and the solution was sonicated for 5 minutes at room temperature to make it homogenously monomeric form. The 31 μL of aliquots was transfered to 2 mL microtubes to make 16 μM final concentration. Samples

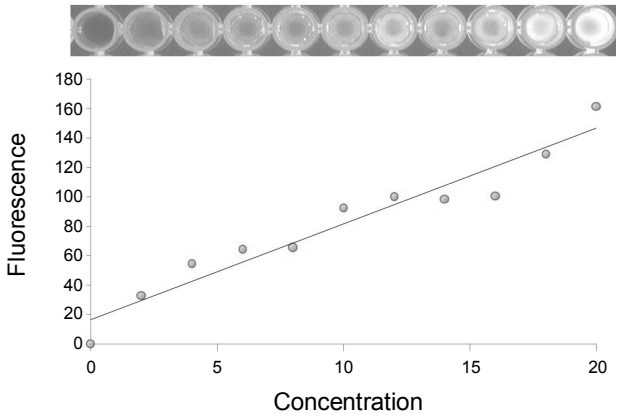

Figure 1. Relationship between the fluorescence intensity and PrP 106-126 concentration.

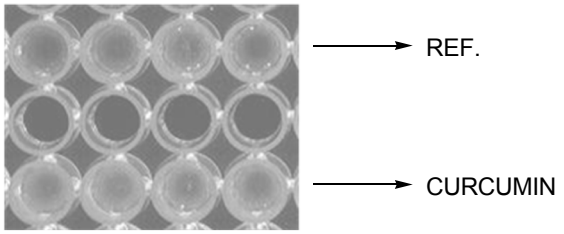

Figure 2. Fluorescence intensity photograph for curcumin's inhibition of PrP 106-126 aggregation and reference data.

are lyophilized and stored in refrigerator. PrP 106-126 aggregation is completed within 1 hour.[19] All samples except samples for standard calibration curve were incubated for 2 hours in 37 °C in a phosphate buffer solution (50 mM, pH 7.50, purchased from Sigma). Buffer solutions were filtered with 0.45 μm Milipore microfilter and autoclaved before use. After incubation, 100 μL aliquot from each sample was filtered through 10,000 Da MW membrane filter (Micropore Microcon centrifugal filter device YM-10) at 10,000 × g for 30 min (Hanil Model Micro 12) to obtain from monomer to pentamer of PrP 106-126. Then, 50 μL filtrates were transferred to a 500 μL microtubes to perform amino acid analysis.[5] After addition of 10 μL of 13.5 M NaOH, the solution was kept at 120 °C autoclave for 2 hours for alkaline hydrolysis of the peptides. After the mixtures were cooled to room temperature, the mixtures were neutralized with methanesulfonic acid (15.4 M, 7.5 μL), and then pH of the mixture was adjusted to pH 9.0 with a boric acid solution (0.7 M, 48 μL). After brief voltexing and centrifugation, the 100 μL of mixture was loaded to a 96 well plate (F96 Cert. Maxisorp, Nunc-Immuno Plate). Solution of fluorescamine in acetonitrile (3.0 mg/mL, 10 μL) was loaded to a 96 well plate (F96 Cert. Maxisorp, Nunc-Immuno Plate) and mixed in plate. The relative value of fluorescence of the resulting solution was measured with alpha-imager (Model 1220 INT) and the relative concentration was determined by comparison with standard calibration curve (Fig. 1).

Curcumin's inhibition rate for the aggregation of oligopeptide larger than pentamer was determined by the procedure mentioned above. After curcumin (purchased from Sigma) was dissolved in ethylalcohol (EtOH), the curcumin in EtOH was added to the samples and incubated with PrP 106-126 (PrP 106-126: 16 μM, curcumin: 16 μM, 1% EtOH) (50 mM phosphate buffer, pH 7.50). For getting a reference data, same amount of curcumin was added into PrP 106-126 solution and amino acid analysis was performed by the above mentioned procedure.

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