플로래스카민과 알파이미저를 이용한 PrP 106-126 펩타이드 정량에 관한 연구

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A Study on the Quantification of PrP 106-126 Peptide by Fluorescamine and Alpha-imager

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최근 광우병 파동과 프리온 단백질의 전염으로 인하여 발생되는 것으로 알려진 크로이츠펠트 야콥병에 대하여 폭넓게 연구되면서 생화학계에서 프리온 단백질에 대한 관심은 상당하다. 본 연구에서는 프리온 단백질의 일부분인 PrP 106-126의 마이크로 몰농도 단위의 정량분석을 실시하였다. 본 연구에서 플로래스카민은 일차 아민과 반응하여 형광을 띠는 물질로써, 알파이미저는 형광의 세기를 측정하는 기기로써 사용되었다. 따라서 합성된 PrP 106-126으로 플로래스카민과 알파이미저를 이용하여 마이크로 몰농도단위의 정량 분석을 위한 조건을 정립하였으며 이를 통하여 표준곡선을 얻을 수 있었다. 이 방법은 차후 변형 프리온 단백질에 대한 응집저해제 및 의약품 연구에 큰 기여를 할 것이다.

Recent social interests on mad cow disease and Creutzfelt-Jacob disease lead researcher's focus onto pathogenic prion proteins causing those diseases. The purpose of this study is to introduce a novel method for micromolecular level quantification of PrP 106-126 peptide, which is a part of prion protein. Fluorescamine has been chosen due to its fluorescence emission characteristics upon reaction with primary amines and Alpha-imager is used to detect the intensity of fluorescence. We succeeded in setting optimal conditions for quantification of PrP 106-206, amyloidogenic prion peptide, at micromolecular level. This study will contribute to identify prion protein aggregation inhibitor and develop new prion protein drug.

Keywords: PrP 106-126, fluorescamine, alpha-imager

1. Introduction

Proteins play important key roles in living organisms. The properties of proteins are significantly changed by the different protein sequence. Proteins have critical functions to bind specific molecules or ions and transfer to other organs, behave as chemical messengers to change cell's activities, and work as antibodies or regulatory proteins.

Total protein quantification is a conventional method used in many scientific researches. There are several protein determination methods that are rapid and accurate for the estimation of protein concentration: a) Bradford Protein Concentration Assay (Coomassie blue G250 dye binding to arginyl and lysyl residues of proteins)[1], b) Lowry method (Folin-Ciocalteau reagent with phenolic group of amino acids (tyrosine and tryptophan residues) produce a blue purple color complex, with maximum absorption in the region of 660 nm wavelength)[2-5], c)

method based on ultraviolet light absorption at 280, 205 nm for aromatic region and peptide bond in amino acids concentration respectively[6], d) Biuret protein assay[7], and e) Bicinchoninic Acid (BCA) Protein Assay (Smith)[6].

Prion diseases and transmissible spongiform encephalopathies (TSEs) are lethal neurodegenerative disorders (Table 1).

Prion diseases are transmissible between animals and humans by inoculation or dietary exposure. Prion is very unique because it is a sole transmissible agent among non-living materials. Prion's pathogenesis is mainly due to conformational rearrangement of a normal cellular prion protein, PrP^C, to an abnormal isoform, PrP^{Sc}. Prion diseases show the following histopathological symptoms: spongiform brain, neuronal loss, astrocytic and microglial proliferation, and prion protein accumulation in the brain, sometimes amyloid deposit formation[9].

Human prion diseases include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), fatal familial insomnia (FFI) and kuru[10,11] (Table 1). Progressive dementia is generally

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Table 1. Prion Diseases of Humans and Animals[8]

Prion disease	Host	Etiology
Scrapie	Sheep and goats	Ingestion, horizontal transmission
Transmissible mink encephalopathy	Mink	Ingestion
Chronic wasting disease	Mule deer, white-tailed deer, Rocky Mountain elk and moose	Ingestion, horizontal transmission
Bovine spongiform encephalopathy	Cattle	
Feline spongiform encephalopathy	Domestic and captive wild cats	Acquired by ingestion of BSE-contaminated meat
Exotic ungulate encephalopathy	Nyala, oryx and greater kudu	
Sporadic	CJD Human	Unknown
Iatrogenic CJD	Human	Acquired by accidental exposure from medical contact with human prion-contaminated neurosurgical instruments, tissue grafts and pituitary hormones derived from human cadavers
Familial CJD	Human	
Fatal familial insomnia	Human	Inherited; all associated with PRNP-coding mutations
Gerstmann-Sträussler-Scheinker disease	Human	
Variant CJD	Human	Environmental exposure possibly via ingestion of BSE-contaminated meat. Three blood transfusion associated cases (from vCJD donors)
Kuru	Human	Exposure via endocannibalism in a small area of Papua New Guinea
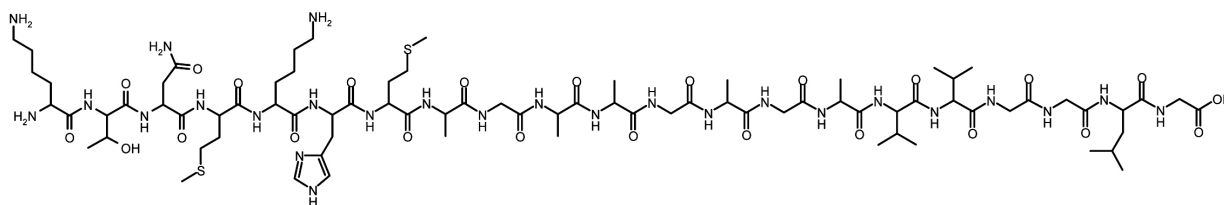
Figure 1. Neurotoxic synthesized prion peptide fragment PrP 106-126[18].

observed in patients having those human prion diseases. Sporadic prion diseases are unique because they are infectious and can be inherited. Approximately 85% of CJD cases occur sporadically with no known cause, whereas 15% of human prion diseases (including GSS, familial CJD and FFI) are suspect to represent autosomal dominantly inherited conditions associated with mutations in the prion protein gene, prion protein[12]. Variant CJD (vCJD) was found in the mid 1990's in the UK[13]. Clinical, neuropathological and molecular evidences support the fact that vCJD is caused by the same prion causing BSE[14-16]. Accumulation of insoluble and protease-resistant PrPSc prion was observed in the central nervous system (CNS) of vCJD patients.

The synthetic peptide PrP 106-126 (KTNMKHMAGAAAAGAVVGGLG) has been known to have the properties of fibrillogenicity and toxicity to neurons in vitro. It shares common features with PrP^{Sc} in terms of biological properties including β-sheet rich formation, protease digestion resistance, insoluble property, and activation of astroglial and microglial cells in vitro. Its mechanism causing cell's death is very similar to PrP^{Sc}, which transforms non-infectious PrP^{C} to infectious PrP^{Sc} [17] (Figure 1).

Determination of amyloidogenic proteins' concentration is mainly conducted by turbidity measurement: Thioflavin-T assay, UV-CD spectroscopy assay, and immunoblotting assay[19,20]. The remaining protein dissolved in solution can be measured by UV-Vis spectrometry at 280 nm[21].

Recently, researchers have focused their efforts on developing drug that is capable of cleaving prion disease relating proteins to cure those amyloidogenic diseases. It has been known that cleaved part of prion protein is not any longer toxic and infectious. Therefore, it is critical to know what kinds of agents can cleave prion disease relating proteins. In a future, discovery of cleavage agent for fatal prion diseases (including human prion diseases, like GSS, familial CJD, and FFI) will be one of hot research topics.

In this study, quantification of total PrP 106-126 was successfully performed.

2. Experimental Section

2.1. Instruments

Synthesized PrP 106-126 (more than 95% purity) is purchased in Peptron Inc. (www.peptron.com). Fluorescamine, methanesulfonic acid, hexafluoroisopropanol, and phosphate buffer powder are purchased in Sigma-aldrich. 96 well plates are purchased from F96 Cert. (Maxisorp, Nunc-Immuno Plate).

2.2. Experiments

2.2.1. Preparation of protein

PrP 106-126 (1 mg) was dissolved in hexafluoroisopropanol (HFIP) with 5 minutes sonication. Then, 20 μL of aliquot was transferred to 500 μL microtubes to make 20 μM final concentration for amino acid analysis. HFIP was added to microtubes, then 20 μL samples from 2 μM to 20 μM concentration range with 2 μM interval were prepared (2 samples for each concentration, total 20 microtubes and 2 references). After HFIP was completely evaporated in heating block (80 ℃, 20 min), phosphate buffer was added to form pH 7.5 final solution.
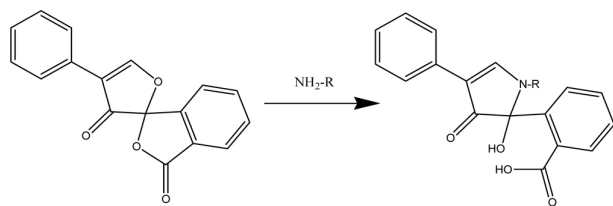
Figure 2. Fluorescamine reaction with primary amine.
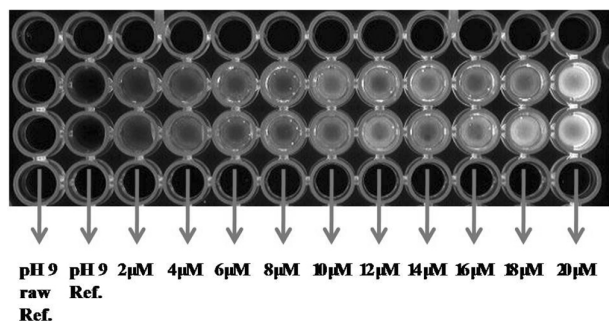
Figure 3. Fluorescence intensity linear to concentration (Raw Ref. is pH 9 buffer + fluorescamine, Ref. is pH 9 buffer + autoclave + fluorescamine).
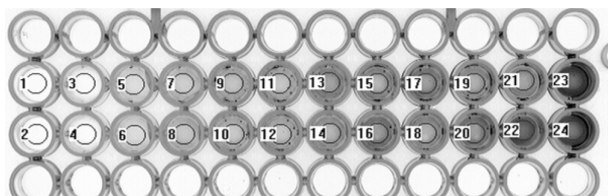
Figure 4. Fluorescence reverse image and intensity circles for measuring density in circles.

2.2.2. Alkaline hydrolysis

For alkaline hydrolysis of PrP 106-206, 10 μL of 13.5 M NaOH was added. Then, microtubes were vortexed and centrifuged for 1 min at 10000 RPM. After 2 h of autoclaving at 120 ℃, the mixtures were then cooled to room temperature[22].

2.2.3. Neutralization

The sample solution containing hydrolyzed PrP 106-126 protein is strongly basic. After methanesulfonic acid 7.5 μL was added, sample vials were vortexed and centrifuged. Finally, after boric acid (0.7 M, 48 μL) was added to make pH 9 in microtubes, samples were vortexed and centrifuged.

2.2.4. Fluorescamine assay

The protein was hydrolyzed and broken down to amino acids and then pH was adjusted to around 9.0. The concentrations of hydrolyzed amino acids are linearly proportional to initial amounts of prion protein. At pH 9, fluorescamine reacts with primary amines within a second at room temperature. Primary amines are known to be appropriate substrate for fluorescamine assay[23,24] (Figure 2). After solution of fluorescamine in acetonitrile (3.0 mg/mL, 10 μL) was loaded
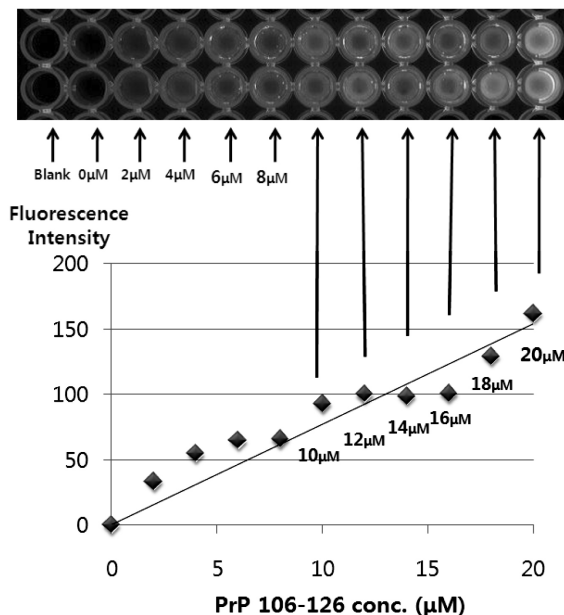
Figure 5. Standard curve for PrP 106-126 by fluorescamine assay.

to a 96 well plate. Then, 85.5 μL of the hydrolyzed mixture was added and mixed in plate. The relative value of fluorescence of the resulting solution was measured with an imaging instrument (Alpha-Imager Model 1220 INT) (Figures 3, 4).

3. Results & Discussion

In this study a novel quantification method to determine total PrP 106-126 protein by using fluorescamine and alpha-imager was successfully developed. After alkaline hydrolysis of the protein, samples are neutralized with acid and buffer. There is linear correlation between hydrolyzed amino acids concentration and initial amount of protein. Amino acid concentration was measured by fluorescence detection followed by reaction with fluorescamine at room temperature. Fluorescence intensity in 96 well plates was measured by alpha-imager. The fluorescence intensity is imaged by alpha-imager and the relative intensities are compared in identical drawn circles. Meaningful correlation between PrP 106-126 and fluorescence upon the reaction of fluorescamine with cleaved primary amines from PrP 106-206 was observed (Figure 5).

There have been a lot of studies to develop drugs that can inhibit the aggregation of prion protein. The cleavage of pathogenic oligomers of amyloidogenic proteins have been widely studied as a new therapeutic options for amyloidogenic diseases[22,25-27]. To enhance our know ledges for the discovery of prion protein drug, it is critical to quantify protein accurately. In this study, quantification for PrP 106-126 at micromolecular level was successfully carried out. This study will contribute to identify prion protein aggregation inhibitor and develop new prion protein drug.

Acknowledgements

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