Search for a highly amyloidogenic peptide region of β-Lactoglobulin

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1 Introduction

Amyloidosis is now a common pathological symptom of more than 20 types of fatal diseases such as Alzheimer's disease, Type II diabetes, and the transmissible spongiform encephalopathies (TSEs) and Creutzfeld-Jakob disease (Selkoe 2003)[1, 2].

These symptoms were mainly caused by the proteins which do not form native structure but irregular structure named "fibrillar aggregates". Interestingly, these fibrillar aggregates were not limited for disease-related proteins. Previous reports have shown that some proteins irrelevant to amyloidosis could form fibrillar aggregates. The most famous example is Bovine β-Lactoglobulin (β-LG)[3]. β-LG is one of the major components of the whey of cow's milk and is apparently a non-disease-related protein. The β-LG forms gels in solution under specific condition, such as exposure to high temperature and high hydrostatic pressure and addition of chemical denaturants. Although there is no structural similarity between native structures of disease-related proteins and non-disease related proteins, atomic force micrographs revealed that structure of heat-induced gels of β-LG is similar to that of disease-related fibrillar aggregates. Therefore, we assumed that there must be common region (factor) to form gels or/and fibrillar aggregates. Many studies about mechanism of amyloid formation revealed that each protein contains the region with high amyloid forming propensity. For example, studies about proteolytic fragments of β_2-microglobulin suggested that K3 peptide (Ser20-Lys41) region of the protein is the essential region to form amyloid fibril[4, 5]. If the mechanism of gel and amyloid fibril formation is the same, it is thought that the same polypeptide region in β-LG is essential for gel and amyloid fibril formation. In this study, we performed analytical experiments to identify a highly amyloidogenic peptide region of β-LG.

2 Materials and Methods

Materials

β-Lactoglobulin (β-LG), crystallized for three times followed by lyophilized and known to be a mixture of variants A and B, was purchased from Sigma (lot no. 033K7003). The molecular weight is 18,400. Aqueous solutions of β-LG were prepared by dissolving β -LG in distilled water. The concentrations were C = 1mM. The pH of the solutions was adjusted by adding HCl aqueous solutions. The protein concentrations were determined by UV spectrometry using an extinction coefficient of 0.961/cm $g^{-1.27}$.

Preparation and characterization of amyloid fibrils

β-LG amyloid fibrils were prepared by incubating protein samples (1 mM) in 20 mM HCl and at pH 2.5 and 80 ℃ for up to 24 hours. In order to confirm the presence of aggregates/fibrils, aliquots of the samples were examined by the application of dynamic force microscopy (DFM), which is a dynamic operation mode of atomic force microscopy (Seiko instruments Inc., SPA400) eliminates the tip-sample repulsive contact.

The identity of amyloid fragments was assessed by mass spectrometry (MS) and N-terminal amino acid sequencing. Analysis of peptidic materials was carried out on a MALDI-Tof mass spectrometer (Voyager DE RP, Applied Biosystems Inc.).

Protein sequence analysis was performed by Edman degradation using an Applied Biosystems protein sequencer model 492HT.

Proteolytic degradation of BLG and isolation of peptide fragment

β-LG was digested with lysyl endopeptidase from *Achromobacter lyticus* (*Achromobacter* protease I, Wako Pure Chemical, Osaka, Japan) at a 1:50 enzyme to substrate ratio at pH 9.0 and 37 °C for 24 h. Digests were separated by reverse phase HPLC on a Cosmosil C18 column (C18M 10E) (SHOWA DENKO, Tokyo, Japan).

The running conditions were a 80-min gradient from 5 to 95% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml min^{-1}. Fractions exhibiting intense peaks

were collected and identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (PerSeptive Biosystems) followed by amino acid composition analysis. Moreover, N-terminal amino acid sequence determination was performed.

Fibril formation based on the identified peptide fragment

The spontaneous fibril formation of the identified fragment of β-LG (15-60) was examined under similar solvent conditions (pH 2.5 and 37°C, at pH 7.0 and 57°C) to those for fibril formation of the whole β-LG. Peptide concentration was adjusted to 100 μM.

3 Results and Discussion

Identification of amyloid fibril's core peptide region by degradation of β-LG at low pH

Amyloid fibrils of β-LG were obtained by incubating concentrated solutions of the protein (1 mM) in 20 mM HCl (pH 2.5) at 80°C for up to 24 hours. Following this procedure, β-LG formed aggregates and AFM observation revealed that the structure of the aggregation included long, straight and unbranched amyloid fibrils (Fig.1). In order to obtain amino acid sequence information of these amyloid fibril, monomers of amyloid fibrils were collected and were identified by mass spectrometry and amino acid sequence analysis.

These analyses revealed that the monomer exhibited molecular weight of 3552.56 Da, and corresponded to the amino acid sequences of Leu1-Asp33 peptide. This result suggested that Leu1-Asp33 peptide must be a core region to be involved in the amyloid fibril formation. Furthermore, Leu1-Asp33 peptide region was highly hydrophobic region. Therefore this region at Leu1-Asp33 position possesses high propensity to amyloid fibril formation.

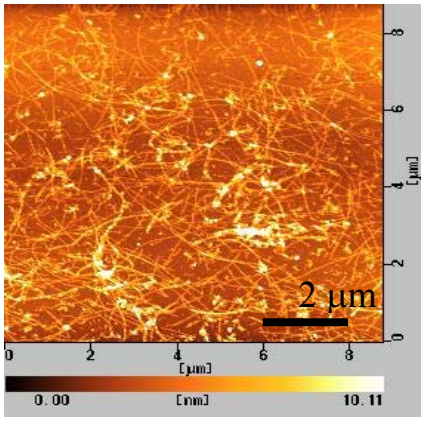

Fig.1 β-LG aggregation at pH 2.0. A, Electron micrograph of β-LG fibrils obtained after 24 hours incubation at pH 2.5 and 80°C.

Proteolysis of β-LG with *Achromobacter* protease I

β-LG was digested with *Achromobacter* protease I to determine highly amyloidogenic peptide fragments. *Achromobacter* protease I was selected because this protease has higher substrate specificity and activity than any other proteases. The proteolytic reaction mixtures were analyzed by RP-HPLC. The peak fractions with high fluorocence absorbance were collected and were identified by molecular mass and amino acid sequence determination. As a result, these analyses revealed that molecular masses of collected fraction was 5000.27 Da corresponding to the Val15-Lys60 region of β-LG.

Fibril formation by the fragment Val15-Lys60

Amyloidogenic properties of the Val15-Lys60 peptide was examined. The observation by AFM images revealed that the region of Val15-Lys60 peptide formed amyloid fibril at neutral pH (Fig.2). The structure and shape of fibrils were similar to those of intact β-LG amyloid fibril suggesting that β-LG formed the amyloid fibril even at neutral condition (pH7.0) after proteolytic cleavage. These results suggested that Val15-Lys60 peptide position of β-LG was important region for amyloid fibril formation. Furthermore, it was concluded that the amino acid peptide at Val15-Asp33 position would be the most important region to form amyloid fibril, since Leu1-Asp33 peptide and Val15-Lys60 peptide have a common region.

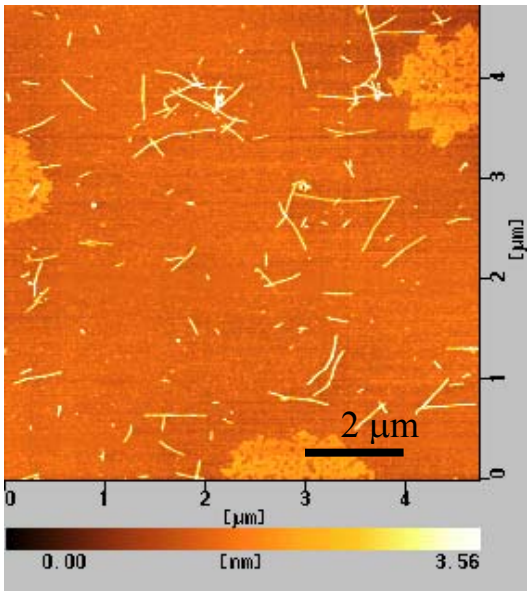

Fig.2
Atomic force micrograph of fibrils from fragment 15–60 obtained after 9 days of incubation at pH 7.0, 57°C

References

1) Selkoe, D.J. (1997) Alzheimer's disease: genotypes, phenotypes, and treatments. *Science*. **275**:630-631.

2) Xing, Y. etal (2002) Amyloid fibril proteins Mech. *Ageing. Dev*. **123**:1625-1636.

3) Walraj, S. Gosal, Allan, H. Clark, Paul, D. A. Pudney, and Simon, B. Ross-Murphy* (2002) Novel Amyloid Fibrillar Networks Derived from a Globular Protein: β-Lactoglobulin

4) Kozhukh, G.V., Hagihara, Y., Kawakami, T., Hasegawa, K., Naiki, H., Goto, Y. (2002) Investigation of a peptide responsible for amyloid fibril formation of beta 2-microglobulin by achromobacter protease I. *J. Biol. Chem.* **277**:1310-1315.

5) Jones, S., Manning, J., Kad, N.M., Radford, S.E. (2003) Amyloid-forming peptides from β2-microglobulin-Insights into the mechanism of fibril formation in vitro. *J. Mol. Biol*. **325**:249-257.