ORIGINAL ARTICLE

# Cytotoxicity of amyloid fibrils derived from hen lysozyme with different properties on SH-SY5Y and cytoprotective effect of *prunus mume* extracts

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**Abstract**

Protein misfolding is associated with formation of amyloid fibrils. Hen egg lysozyme, well-known for its structural properties, has been shown by atomic force microscopy to form needle-shaped amyloid fibrils under acidic conditions with high temperature. When disulfide bonds of hen lysozyme were reduced by reductant TCEP, it formed noodle-shaped amyloid fibrils. Difference in thioflavin T and 8-Anilino-1-naphthalenesulfonic acid (ANS) fluorescence of these fibrils suggested difference in structural properties. The needle-shaped amyloid fibrils exerted cytotoxicity on human neuroblastoma SH-SY5Y cells, but noodle-shaped amyloid fibrils did not. The reason for cytotoxicity being exerted only by needle-shaped amyloid fibrils was thought to be due to the difference in properties, such as fibril surface and inter-/intra-molecule structure. This finding indicates the importance of studying the interaction between the amyloid fibril surface and inter-/intra-molecular structural properties and cells. We also investigated whether *Prunus mume* extracts protect SH-SY5Y cells from hen lysozyme amyloid-induced toxicity. The *Prunus mume* seed extract protected the cells from needle-shaped amyloid toxicity. *Prunus mume* could therefore potentially prevent amyloid-related disease.

## INTRODUCTION

Alzheimer's disease and Parkinson's disease are associated with the misfolding of peptides or proteins and the subsequent formation of ordered amyloid fibrils (Cohen, 2015). The ability of normally soluble proteins to convert into amyloid fibrils is now recognized to be a generic phenomenon (Dobson, 2017). Different proteins have unique three-dimensional structures determined by their amino acid sequences and they thus perform various different functions. The core elements of amyloid derived from such proteins form a similar cross-β structure. To better understand medical disorders such as Alzheimer's disease and Parkinson's disease, it is important to study amyloid formation of general protein and amyloid toxicity.

Human lysozyme amyloid deposits are observed in patients with familial lysozyme systemic amyloidosis (Pleyer, 2015). Hen egg white lysozyme has a high degree of structural homology with human lysozyme and has four disulfide bonds (Mine, 2000). It is one of the most studied model proteins that forms amyloid fibrils by reducing the protein stability under highly acidic conditions and high temperatures (Arnaudov, 2005; Shah, 2012; Sasaki, 2008). All disulfide-deficient variants of hen lysozyme (0SS) are intrinsically unfolded and spontaneously associate to form amyloid-like fibrils under mildly acidic conditions without denaturant (Niraula, 2004). These amyloid fibrils show different structural morphology under atomic force microscopy. The exact mechanism of amyloid toxicity remains unclear, so better understanding of amyloid fibril formation and amyloid toxicity could be important in treatment and prevention of misfolding diseases. Here, we compare the structural morphology and toxicity of amyloid fibrils formed from wild-type hen lysozyme, the stability of which was reduced by high temperature in the presence or absence of reductant.

Protecting cells from amyloid toxicity leads to the prevention of amyloid-related diseases. We have studied the effects of *Prunus mume*, a traditional food in Japan, on various diseases and overall health (Kono, 2011, 2014, 2018). *Prunus mume*, called "*ume*" in Japanese, is a traditional food and medicine

in Asian countries such as Japan and China. *Prunus mume* seed components are contained in various processed *Prunus mume* products, including being dried or pickled with salt, and are an ingredient in liquors and soft drinks, so we used *Prunus mume* seed extracts in this research. We examined *Prunus mume*-derived components that prevent amyloid toxicity using amyloid fibrils that exert cytotoxicity.

## MATERIALS AND METHODS

### Amyloid fibrillation

For amyloid fibrillation, hen egg white lysozyme (HEWL, 4 mg/ml, Seikagaku Corporation, Tokyo, Japan) was incubated at 57°C, transition temperature (Sasaki, 2008), for indicated periods in 20 mM glycine-HCl (Gly-H) buffer, pH2.2, with 80 mM NaCl and supplemented with or without reductant Tris (2-carboxyethyl) phosphine Hydrochloride (TCEP, Thermo Fisher Scientific, Waltham, MA, USA).

### Thioflavin T and ANS fluorescence

Amyloid fibrillation was detected by Thioflavin T (ThT) fluorescence. Incubated HEWL solution (60 μl) was mixed with 540 μl of 20 mM glycine-HCl buffer supplemented with 80 mM NaCl and 20 μM ThT. ThT fluorescence emission spectra of diluted solution were taken with an excitation wavelength of 440 nm on RF-5300PC fluorescence spectrometer (Shimadzu, Kyoto, Japan). For ANS fluorescence, ten μl of HEWL solution was mixed with 990 μl of buffer supplemented with 2.8 μM ANS (Sigma-Aldrich, St. Louis, MO, USA). ANS fluorescence emission spectra of diluted solution were taken with an excitation wavelength of 350 nm.

### Atomic force microscopy

Atomic force microscopy (AFM) observation of amyloid fibril was carried out on a scanning probe microscope SPI-3800 (Seiko Instruments Inc.). One microliter of each of the fibril solutions was taken and diluted 500-fold. Ten microliters of the diluted solution were immediately deposited on the freshly-cleaved mica surface, rinsed with pure water, which was blotted sideways and left to dry. AFM images were recorded under a dynamic-force cyclic-contact mode at a frequency 110 - 150 kHz with a cantilever DF20 (SII NanoTechnology, Chiba, Japan).

### Cell culture

Human neuroblastoma cell line SH-SY5Y (KAC, Kyoto, Japan) was grown at 37°C in a 5% $CO_2$ atmosphere in Dulbecco's Modified Eagle Medium/ Ham's F12 (Life Technologies, Carlsbad, CA, USA) supplemented with 1% non-essential amino acids (Life Technologies), 15% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA).

### Cell viability

SH-SY5Y cells were seeded at a density of $1 \times 10^4$ cells/well in 96-well plates and cultured for 48 hours. Cells were additionally cultured in serum-free medium for 24 hours. Incubated lysozyme solution was dialyzed to remove TCEP and was diluted in culture medium (without FBS) to a final concentration of 0 to 0.4 mg/ml. For cell viability assay, cells were then treated with 100 μl of culture medium including diluted lysozyme solution for 48 hours. After incubation, the medium was replaced with 100 μl of fresh medium, and 25 μl of 3- (4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium, inner salt (MTS; Promega, Madison, WI, USA) solution was added to each well. Cells were then incubated for 1 hour at 37°C in 5% $CO_2$ atmosphere. The absorbance of each well was recorded at 490 nm using a microplate spectrometer (Hitachi High-Technologies Corporation, Tokyo, Japan).

### Extraction and separation from Prunus mume seed

Natural ingredients for experiments were extracted from *Prunus mume* seeds (provided by Okahata Farm, Wakayama, Japan). The extract used was the same as the extract obtained in a previous report (Kono, 2018). Briefly, the seeds were crushed and kernels were removed. The de-kerneled seeds (described hereafter as 'seeds') were used for the experiment. The seeds were extracted with methanol then distilled water-suspended methanol extract was further extracted with hexane, dichloromethane and ethyl acetate. The dichloromethane fraction was subjected to silica gel column chromatography and eluted with dichloromethane/acetone to separate six fractions (Fr. 1～6). Fr. 2 was further subjected to silica gel column chromatography and eluted with hexane/ethyl acetate to separate six subfractions (Fr. 7～12). Fr. 4 was also further subjected to silica gel column chromatography and eluted with hexane/ethyl acetate to separate five subfractions (Fr. 4-1～4-5). Each fraction was dissolved in dimethyl sulfoxide (DMSO) and used to measure the protective effect on amyloid toxicity.

## RESULTS

### Amyloid fibril formation of hen lysozyme in the presence or absence of TCEP

Lysozyme amyloid fibrillation was confirmed by AFM observation (Figure 1). Lysozyme slowly formed needle-shaped amyloid fibrils (Figure 1B, left panel) under high temperature and acidic conditions over a week. When TCEP was added, however, lysozyme quickly formed noodle-shaped amyloid fibrils (Figure 1B, right panel). Similarly, from the change in ThT fluorescence intensity, when TCEP was not contained, the fluorescence intensity increased over one week or longer. When TCEP was contained, however, the fluorescence intensity increased within a few hours (Figure 2A and 2B). The β-sheet structure typical of amyloid structure was revealed to be increased with lysozyme fibril formation on both needle-like and noodle-like shapes.

### Cytotoxicity difference of two types of amyloid fibrils

The cytotoxicity of needle-shaped and noodle-shaped amyloid fibrils against human neuroblastoma SH-SY5Y cells was evaluated by cell viability assay. The cytotoxicity of needle-shaped amyloid fibrils (TCEP-) increased in a concentration-dependent manner (Figure 3B), but that of noodle-shaped amyloid fibrils (TCEP+, Figure 3C) and monomeric state of lysozyme (Figure 3A) did not show cytotoxicity. The cell viability was decreased with increasing incubation period of TCEP- (Figure 3D). Slight increase of cell viability was observed in the treatment of noodle-shaped amyloid fibrils.

### ANS fluorescence difference between two types of amyloid fibrils

8-Anilino-1-naphthalenesulfonic acid (ANS) binds to proteins through hydrophobic and electrostatic interactions to increase fluorescence intensity (Su-

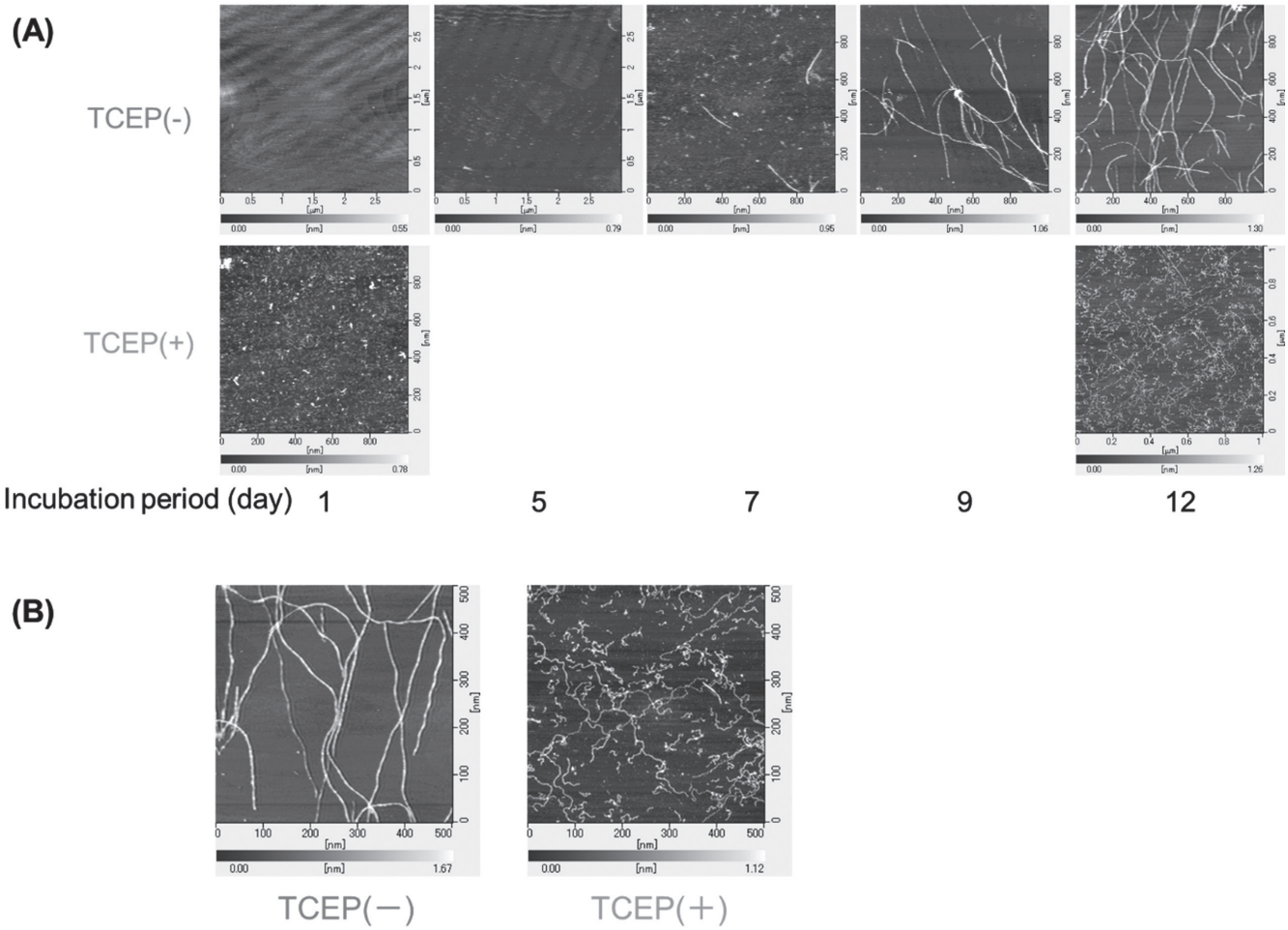


**Figure 1.** AFM images of Lysozyme amyloid fibrils. (A) AFM images show lysozyme incubated with (+) or without (-) TCEP for the indicated period. (B) Higher magnification AFM images of lysozyme incubated with (+) or without (-) TCEP for 12 days.

latsky, 2020). The ANS fluorescence of lysozyme incubated without TCEP (TCEP-) increased throughout the incubation period (Figure 4). Meanwhile, the ANS fluorescence intensity of lysozyme incubated with TCEP (TCEP+) increased early, but its maximum fluorescence intensity was 1/7 lower than that of TCEP (-).

**Protective effect of Prunus mume extract on cytotoxic amyloid-induced cell death**

Cytotoxic amyloid (TCEP+) was used to investigate the *Prunus mume* seed-derived component that protects cells from toxicity. The protective effect against cell death was induced by the lysozyme amyloid treatment, a cytoprotective effect was observed

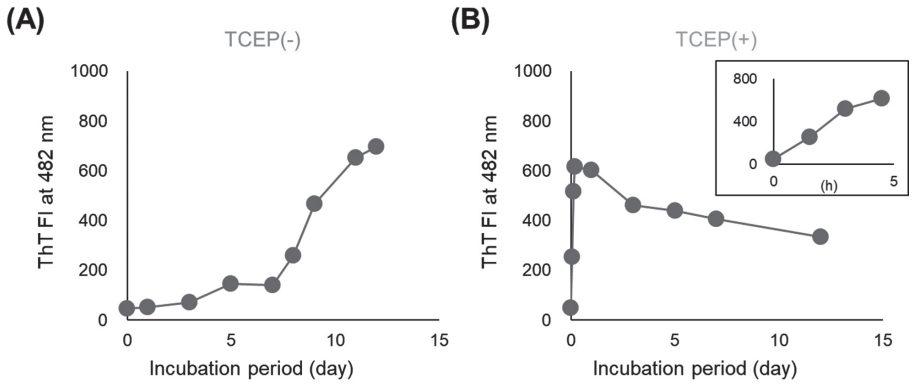


**Figure 2.** ThT fluorescence changes on Lysozyme amyloid fibrillation and the effect of lysozyme amyloid fibrils on SH-SY5Y cell viability. (A, B) ThT fluorescence intensity (a.u.) at 482 nm of lysozyme incubated with (+) or without (-) TCEP were plotted against indicated period of incubation. Right panel (B) includes data of the ThT fluorescence changes in early reaction.

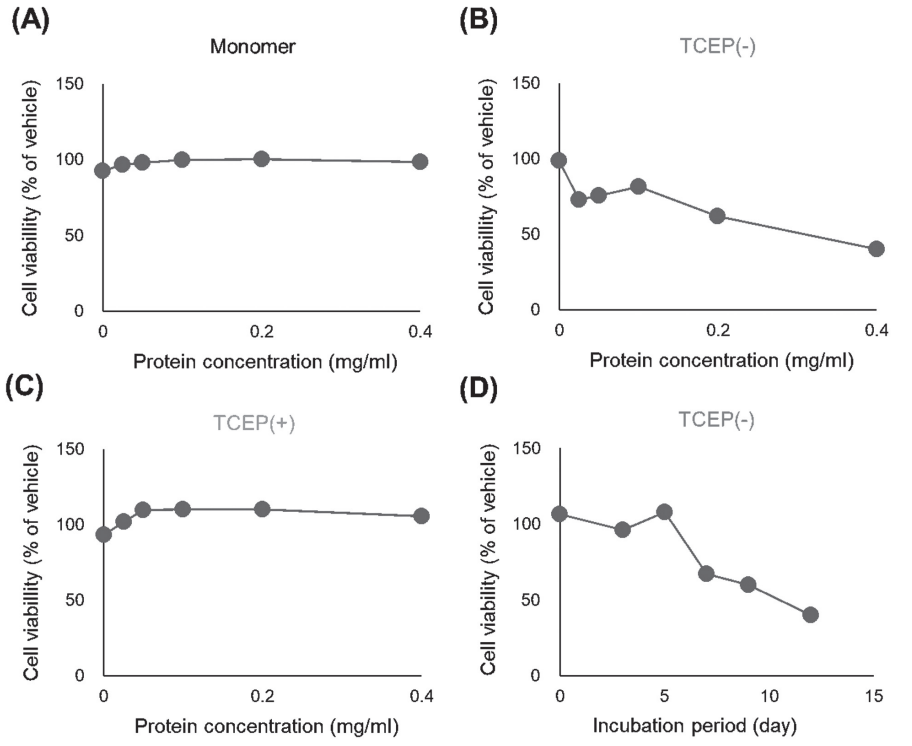


**Figure 3.** Concentration dependent effect of Lysozyme amyloid fibrils formed from incubation with (+) or without (-) TCEP on SH-SY5Y cell viability. Cells were treated with monomeric state of lysozyme (A), 12-day incubated lysozyme without TCEP (B) or 12-day incubated lysozyme with TCEP. Final lysozyme concentrations were 0～0.4 mg/ml. (D) Effect of different incubation period of lysozyme incubated without TCEP on cell viability. Cells were treated with 0.4 mg/ml of lysozyme. Cell viability was expressed as % of vehicle (buffer alone) treatment (n=2).

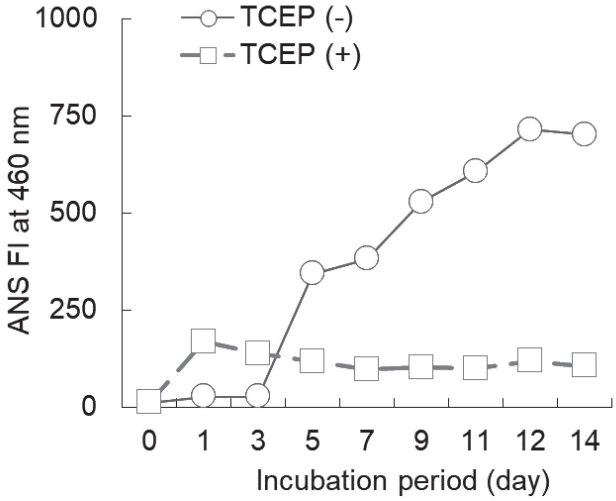


**Figure 4.** ANS fluorescence intensity change with incubation period of lysozyme incubated with or without TCEP. ANS fluorescence intensity (a.u.) at 460 nm of lysozyme incubated with (+) or without (-) TCEP were plotted against indicated period of incubation.

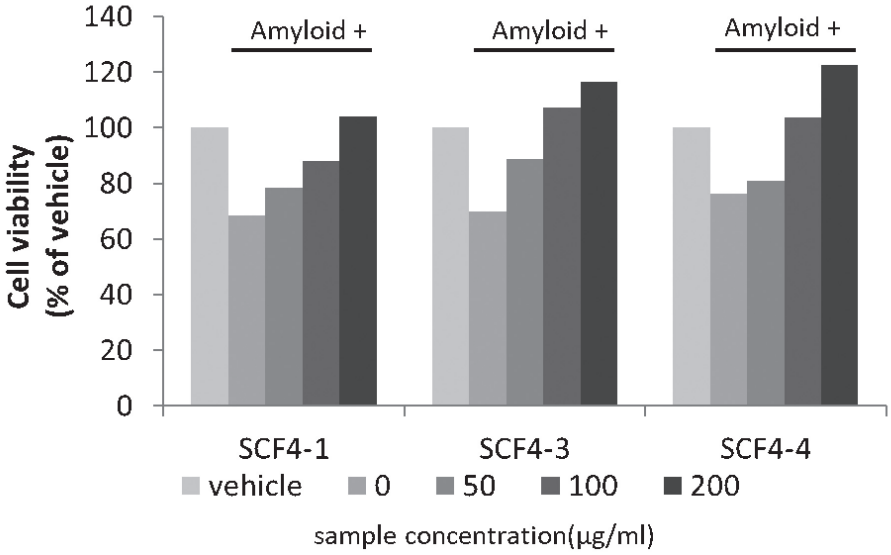


**Figure 5.** Protective effect of *Prunus mume* seed extracts on cytotoxicity induced by toxic lysozyme amyloid fibril. After incubation of SH-SY5Y cells in serum free medium for 24 h, cells were treated for 48 h with 12-day incubated lysozyme and indicated concentrations of *Prunus mume* seed extracts. Cell viability, absorbance of MTS at 490 nm, was expressed as % of vehicle (buffer alone) treatment (n=2).

in fractions Fr. 4-1, 4-3, 4-4 (Figure 5). Cell viability was recovered with the increase of concentrations of these fractions.

## DISCUSSION

To better understand medical disorders including Alzheimer's disease and Parkinson's disease, we studied amyloid formation of hen lysozyme and amyloid toxicity. Lysozyme formed needle-like fibers under the high temperature and acidic conditions adopted in this study. Under such conditions, the population of folded state of lysozyme partially remains (Sasaki, 2008). When TCEP is added, however, the disulfide bonds are reduced, and the structural stability is greatly reduced, similar to the case of 0SS, and we speculate that the population of the unfolded state is increased.

Hen lysozyme has four disulfide bonds. A study of amyloid fibrillation of four molecular species of

hen lysozyme single-disulfide variants, which have only one of the four known disulfide bonds, indicated that the location of disulfide bonds is a factor in amyloid fibrillation rate (Kono, 2009). In particular, the disulfide bond Cys6-Cys127, bonding the N-terminal and the C-terminal regions, strongly inhibits amyloid fibrillation compared with 0SS. In contrast, disulfide bonds Cys30-Cys115 or Cys64-Cys80, known to be important for the formation of native secondly structures, enhance amyloid fibrillation. Specifically, disulfide bonds, which are important for stabilizing the native structure, may be favored in fibrillation. 0SS, all-disulfide deficient hen lysozyme, has been reported to form noodle-shapes and short amyloid-like protofibrils (Abdul Latif, 2007; Niraula, 2004) while wild-type forms needle-shapes and long amyloid fibrils (Sasaki, 2008; Shah, 2012).

Lysozyme was shown to form needle-shaped amyloid fibrils by reducing the stability under highly temperature without TCEP. In contrast, lysozyme with TCEP to reduce disulfide bonds formed noodle-shaped amyloid fibrils similar to 0SS. The needle-shape amyloid fibrils (TCEP-) showed cytotoxicity to SH-SY5Y cells, but noodle-shape amyloid fibrils (TCEP+) did not show cytotoxicity. The difference in ThT and ANS fluorescence of both TCEP- and TCEP+ indicates that the differences in the structural properties of TCEP- and TCEP+ are the amount of β-sheet structure, hydrophobicity and charge of fibril surfaces. Previous reports have shown that 0SS fibril is highly voluminous and in a compressible state (Akasaka, 2007; Abdul Latif, 2007), meaning it has considerable cavities, but the properties of wild-type lysozyme amyloid fibril induced without denaturant or reductant seem to be different from those of 0SS fibril (Shah, 2012). Similarly, TCEP- and TCEP+ are expected to have different *intra*molecular and *inter*molecular structures. The difference in cytotoxicity, of TCEP- and TCEP+ including interaction with cells, is suggested to be influenced by the difference in the morphology of the amyloid fibrils, the structural properties of *intra*- and/or *inter*-molecule and the structural properties of the amyloid fibril surface. In particular, biophysical properties such as charge and hydrophobicity of fibrils are presumed to greatly affect the interaction with the cell membrane. Detailed mechanisms on the interaction between membrane and amyloid remain unclear, but some studies have indicated that the interaction between fibrils or oligomers and the biological membrane causes membrane disruption and destabilization (Sciacca, 2018; Smith, 2015). The difference in cytotoxicity of lysozyme amyloid fibrils having different morphological and structural properties is therefore suggested to be mainly due to the different interaction between the cell membrane and the fibrils, that is, the different charge and hydrophobicity of the fibrils. To further elucidate the toxicity mechanism of amyloid, it is important to study the interaction between the amyloid fibril surface and inter-/intra-molecular structural properties and cells.

Finally, we investigated whether *Prunus mume* extracts protect SH-SY5Y cells from hen lysozyme amyloid-induced toxicity. Fractions found to prevent amyloid-induced cytotoxicity include some lignan compounds such as lyoniresinol. Lignans are known to possess various pharmacological properties, notably, some lignans have a neuroprotective effect (Sowndhararajan, 2018). Although the detailed composition and mechanisms are unknown, the finding that *Prunus mume* seed extract protects cells from amyloid toxicity is important for future dementia research.

Needle-shaped and noodle-shaped hen lysozyme amyloid fibrils have differential properties, such as the apparent form of the fibrils, the amount of β-sheet, the fibril surface and the inter-/intra-molecule structure. We suggest the reason for only needle-shaped amyloid fibrils exerting cytotoxicity was due to the difference in these properties. In addition, *Prunus mume* seed extracts may have the potential to protect the cells from needle-shaped amyloid toxicity.

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