Biochimica et Biophysica Acta 1822 (2012) 1316–1324

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Anti-amyloidogenic effects of soybean isoflavones *in vitro*: Fluorescence spectroscopy demonstrating direct binding to Aβ monomers, oligomers and fibrils

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ARTICLE INFO

Article history:
Received 27 February 2012
Received in revised form 21 April 2012
Accepted 8 May 2012
Available online 14 May 2012

Keywords:
Alzheimer's disease
Amyloid β-protein oligomer
Fragment
Photo-induced cross-linking of unmodified proteins (PICUP)
Glycitein
Three-dimensional fluorescence spectroscopy

ABSTRACT

Alzheimer's disease is characterized by the presence of extracellular deposits of amyloid, primarily composed of the amyloid β-protein (Aβ). A growing body of evidence indicates that oligomeric forms of Aβ play a critical role in disease causation. Soybean isoflavones are flavonoids with an isoflavone backbone. Isoflavones have been reported to protect against Aβ-induced neurotoxicity in cultured cell systems, the molecular mechanisms remain unclear. Our previous studies demonstrated that red wine-related flavonoids with a flavone backbone are able to inhibit Aβ assembly and destabilize preformed Aβ aggregates. Here, we show that isoflavones, especially glycitein and genistein, have anti-fibrillization, anti-oligomerization and fibril-destabilizing effects on $Aβ_{1–40}$ and $Aβ_{1–42}$ *in vitro* at physiological pH and temperature, by using nucleation-dependent polymerization monitored by thioflavin T fluorescence, atomic force microscopy, electron microscopy, and photo-induced cross-linking of unmodified proteins followed by SDS-PAGE. Our three-dimensional fluorescence spectroscopic analyses demonstrated that glycitein interacted with Aβ monomers, oligomers and fibrils, indicating specific binding of glycitein to these Aβ species. Glycitein also interacted with different Aβ fragments ($Aβ_{1–42}$, $Aβ_{1–40}$, $Aβ_{1–16}$ and $Aβ_{25–35}$), exhibiting the highest fluorescence enhancement with $Aβ_{25–35}$. We speculated that glycitein's anti-amyloidogenic properties are specifically mediated by its binding to Aβ monomers, oligomers and fibrils. Isoflavones may hold promise as a treatment option for preventative strategies targeting amyloid formation in Alzheimer's disease.

1. Introduction

Alzheimer's disease is characterized by the presence of intracellular neurofibrillary tangles consisting of hyperphosphorylated tau and extracellular parenchymal and vascular amyloid deposits largely composed of amyloid β-protein (Aβ) [1]. The aggregation of Aβ monomers and β-sheet formation are considered to be critical events rendering these peptides neurotoxic [2]. Moreover, oligomeric forms of Aβ appear to play a crucial role in disease causation [3–7].

Flavonoids are chemically classified into a number of different groups; i.e., flavone, flavonol, flavanone, flavan-3-ol, anthocyanidin and isoflavone (Supplemental Fig. S1A). A substantial body of evidence indicates that flavonoids can inhibit the fibrillization of a variety of amyloidogenic proteins [8–10]. Isoflavones are a chemical class with about 15 members, and are found in high concentrations in soybean, red clover, *Pueraria mirifica* and other plants. Stilbenoids are polyphenolic compounds derived from natural products. Resveratrol is a major stilbenoid (Supplemental Fig. S1B), which has been reported to exert potent neuroprotective effects [11]. Although epidemiological studies on oral consumption of isoflavones for Alzheimer's disease have never been reported, the consumption of red wine, related to resveratrol and flavonoids with the flavone backbone, are associated with a lower risk of dementia [12,13].

Major soybean isoflavones, including glycitein (Gly) and genistein (Gen) (Fig. 1) have been reported to inhibit Aβ-induced apoptosis of cultured cells [14]. Several mechanisms may contribute to their neuroprotective effects: (i) isoflavones inhibit caspase activation, thereby reducing apoptosis [14]; (ii) the antioxidant activity of isoflavones mitigates Aβ-mediated toxicity, which is at least in part, mediated by oxidative injury [14]; (iii) isoflavone-induced arginase 1 activity protects glial cells and promotes axonal regeneration [15].

We previously reported that red wine-related flavonoids with the flavone backbone are effective inhibitors of $Aβ_{1–40}$ and $Aβ_{1–42}$ fibrillization *in vitro*, and that these compounds could also destabilize preformed Aβ assemblies [8]. Our prior work demonstrated that a flavonoid myricetin, with a flavone backbone (Supplemental Fig. S1C),

Abbreviations: Aβ, amyloid β-protein; AFM, atomic force microscopy; AU, arbitrary unit; CD, circular dichroism; CSF, cerebrospinal fluid; Dai, daidzein; DTT, dithiothreitol; EM, electron microscopy; Equ, (±)-Equol; EM, emission; EX, excitation; fAβs, Aβ fibrils; For, formononetin; Gen, genistein; Gly, glycitein; PB, phosphate buffer; PICUP, photo-induced cross-linking of unmodified proteins; Pur, puruneti; SE, standard error; ThT, thioflavine T; TTR, transthyretin protein

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doi:10.1016/j.bbadis.2012.05.006

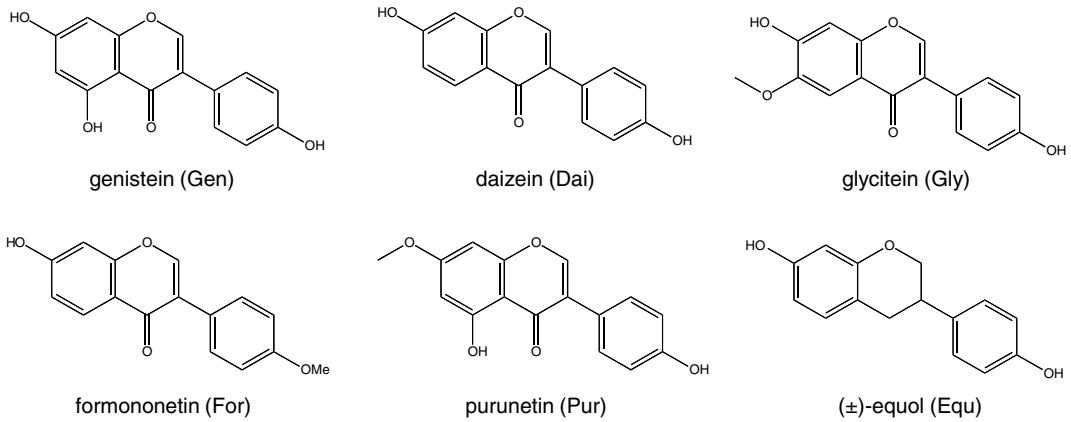


Fig. 1. Structures of isoflavones analyzed in this study.

preferentially and reversibly bound Aβ fibrils (fAβ), rather than monomeric Aβ, revealing a molecular mechanism for the ability of flavonoids to inhibit Aβ-fibrillization and to destabilize preformed fAβ [16]. Furthermore, flavonoids with the flavone backbone have been shown to inhibit pre-protofibrillar oligomerization with photo-induced cross-linking of unmodified proteins (PICUP) [10].

Here, we examined the effects of isoflavones on Aβ-fibrillization, Aβ-oligomerization and preformed Aβ assemblies, *in vitro* at pH 7.4 and 37 °C, using a nucleation-dependent polymerization model [17,18] and PICUP followed by SDS-PAGE [10,19]. Moreover, we examined whether isoflavones could interact with the monomers, oligomers and fibrils of Aβs by using three-dimensional fluorescence spectroscopy.

2. Material and methods

2.1. Preparation of Aβ solutions

$Aβ_{1-40}$ (trifluoroacetate form, lot number 541226; Peptide Institute Inc., Osaka, Japan), $Aβ_{1-42}$ (trifluoroacetate form, lot number 580403, Peptide Institute Inc.), $Aβ_{1-16}$ (HCl form, lot number 570408, Peptide Institute Inc.) and $Aβ_{25-35}$ (trifluoroacetate form, lot number 600102, Peptide Institute Inc.) were handled in a 4 °C cold room. The peptides were dissolved by brief vortexing in 60 mM NaOH–10 mM phosphate buffer (PB) and brought to final concentrations of 500 μM (2.2 mg/mL) and 250 μM, and stored at −80 °C before assaying (fresh $Aβ_{1-40}$, $Aβ_{1-42}$, $Aβ_{1-16}$ and $Aβ_{25-35}$ solutions).

2.2. Peptide fibrillization assay

Fibrillization of $Aβ_{1-40}$ and $Aβ_{1-42}$ was assayed as described [8]. The reaction mixture contained 25 μM $Aβ_{1-40}$ or $Aβ_{1-42}$, and 0, 25 or 250 μM of the isoflavone—Gen, daidzein (Dai), Gly, formononetin (For), purunetin (Pur), or (±)-Equol (Equ) (Sigma Chemical Co., St. Louis, MO, USA) (Fig. 1)—and 10 mM PB (pH 7.4). The isoflavones (Gen, Dai, Gly, For, Pur and Equ) were dissolved in 10 mM PB to a concentration at 2.5 mM, and these were added to the reaction mixture to a final concentration of 25 or 250 μM. Aliquots (100 μL) of the mixture were transferred to 100 μL PCR tubes (Takara Shuzo Co. Ltd., Otsu, Japan) and incubated at 37 °C for 0–7 days without agitation.

2.3. Fluorescence spectroscopy monitored by thioflavine T

Fluorescence spectroscopy was performed using Hitachi F-7000 fluorescence spectrophotometers (Tokyo, Japan) as described [8]. Fluorescence measurements of $fAβ_{1-40}$ and $fAβ_{1-42}$ were obtained at excitation and emission wavelengths of 445 and 490 nm, respectively, with the reaction mixture containing 5 μM thioflavine T (ThT) (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 50 mM of glycine–NaOH buffer (pH 8.5). From each reaction tube, triplicate 5-μL aliquots were removed and subjected to fluorescence spectroscopy, and the mean of the 3 measurements was determined by subtracting the fluorescence of a ThT blank. The concentration of isoflavones in the ThT solution was diluted up to 1/200 of that in the reaction mixture. We confirmed that all our tested compounds did not quench ThT fluorescence at the diluted concentration.

2.4. Chemical photo-induced cross-linking and determination of oligomer frequency distributions

Immediately after their preparation, samples were cross-linked using PICUP, as described [18]. Briefly, to an 18-μL volume of protein solution were added 1 μL of 1 mM tris(2,2-bipyridyl) dichlororuthenium(II) (Ru(bpy)) and 1 μL of 20 mM ammonium persulfate. The final protein:Ru(bpy):ammonium persulfate molar ratios for $Aβ_{1-40}$ and $Aβ_{1-42}$ were 0.29:1:20 and 0.16:1:20, respectively. The mixture was irradiated for 1 s with visible light, and then the reaction was quenched with 40 μL of Tricine sample buffer (Invitrogen, Carlsbad, CA) containing 2 μL of 1 M dithiothreitol (DTT). Determination of the frequency distribution of the monomers and oligomers was accomplished using SDS-PAGE and silver staining, as described [19]. Briefly, 10 μL of each cross-linked sample was electrophoresed on a 10–20% gradient tricine gel and visualized by silver staining (SilverXpress, Invitrogen). Non-cross-linked samples were used as controls in each experiment.

2.5. Size exclusion chromatography

PICUP reagents were removed from cross-linked samples by size exclusion chromatography as described [20]. To do so, 1.5-cm diameter cylindrical columns were packed manually with 2 g of Bio-Gel P2 Fine (Bio-Rad), which produced a 6-mL column volume. The column was first washed twice with 25 mL of 50 mM NH_4HCO_3 (pH 8.5). A 216-μL volume of 50–100 μM cross-linked sample was then loaded. The column was eluted with the same buffer at a flow rate of approximately 0.15 mL/min. The first 1 mL of eluate was collected. The fractionation range of the Bio-Gel P2 column is 100–1800 Da. Thus, Aβ peptides eluted in the void volume, whereas Ru(bpy) ($M_r = 748.6$), ammonium persulfate ($M_r = 228.2$) and dithiothreitol ($M_r = 154.2$) entered the column matrix and were separated from Aβ. Fractions were lyophilized immediately after collection and were reconstituted to the indicated concentration, in 10 mM PB (pH 7.4).

2.6. Cross-linked oligomer-destabilization assay

Destabilization of cross-linked Aβ oligomers was assessed as follows and then subjected to SDS-PAGE followed by silver staining.

Briefly, the reaction mixture contained 25 μM cross-linked $A\beta_{1-40}$ or $A\beta_{1-42}$, 250 μM of the isoflavone and 10 mM PB (pH 7.4) were placed in PCR tubes. Each sample was incubated at 37 °C for 24 h, and dried in a centrifugation evaporator for 12 h at 37 °C. Twenty microliters of SDS sample buffer was added to these dried fractions, and 10 μL of each sample was electrophoresed on a 10–20% gradient tricine gel and visualized by silver staining (SilverXpress, Invitrogen).

2.7. Fibril-destabilization assay

Destabilization of fAβs was assessed as reported previously [8]. Briefly, the reaction mixture contained 25 μM fresh $fA\beta_{1-40}$ or $fA\beta_{1-42}$, 0–250 μM of the isoflavone and 10 mM PB (pH 7.4). Three 5-μL aliquots were subjected to fluorescence spectroscopy and 30-μL aliquots were placed in PCR tubes. The temperature was elevated from 4 to 37 °C. Incubation times ranged from 0 to 24 h, as indicated in each figure, and the reaction was stopped by placing the tubes on ice. The mean of the 3 measurements was determined. At the diluted concentration, these compounds did not compete with ThT for fAβ at either 4 or 37 °C for a period of 1 min.

2.8. Fractionation of the reaction mixture and SDS-PAGE

$A\beta_{1-40}$ and $A\beta_{1-42}$ in the reaction mixtures of $fA\beta_{1-40}$ and $fA\beta_{1-42}$ destabilization were fractionated into phosphate buffer-soluble (PB-sol) and PB-insoluble (PB-insol) fractions and then subjected to SDS-PAGE followed by silver staining, as follows. Aliquots (20 μL) of the reaction mixtures were removed and centrifuged at 21,500 × *g* for 2 h at 4 °C. After removal of the supernatants containing $A\beta_{1-40}$ and $A\beta_{1-42}$ soluble in phosphate buffer (PB-sol fraction), the pellets were independently dried in a centrifugation evaporator for 6 h at 37 °C. Then, 20 μL of 73% formic acid was added to the pellets containing $A\beta_{1-40}$ and $A\beta_{1-42}$ insoluble in PB (PB-insol fraction), vortex-mixed, incubated at room temperature for 1 h, and dried in a centrifugation evaporator for 12 h at 25 °C. The PB-sol fraction was dried in a centrifugation evaporator for 12 h at 25 °C. Twenty microliters of SDS sample buffer was added to these dried fractions, and 6 μL of each sample was electrophoresed on a 10–20% gradient tricine gel and visualized by silver staining (SilverXpress, Invitrogen).

2.9. Electron microscopy (EM)

A 20-μL aliquot of each sample was spotted onto a carbon-coated grid (Okenshoji, Co., Ltd., Tokyo, Japan) and incubated for 5 min. The peptide was stained negatively with 1% phosphotungstic acid (pH 7.0). This solution was wicked off, and then the grid was air-dried. Samples were examined using a JEM-1210 transmission electron microscope with an acceleration voltage of 75 kV.

2.10. Atomic force microscopy (AFM)

Peptide solutions were characterized using a Nanoscope IIIa controller (Veeco Digital Instruments, Santa Barbara, CA) with a multimode scanning probe microscope equipped with a JV (J-type vertical) scanner. All measurements were carried out in the tapping mode under ambient conditions using single-beam silicon cantilever probes. A 10-μL aliquot of each lyophilized peptide, reconstituted to a concentration of 25 μM in 10 mM PB (pH 7.4), was spotted onto freshly cleaved mica (Okenshoji Co. Ltd., Tokyo, Japan), incubated at room temperature for 5 min, rinsed with water, and then blown dry with air. The sample was analyzed by examining at least five regions of the mica surface to confirm the homogeneity of the structures throughout the sample.

2.11. Three-dimensional fluorescence spectroscopic characterization of isoflavone emission spectra

Fluorescence emission of isoflavones in the presence or absence of the various Aβ forms was characterized as follows. The reaction mixtures (200 μL), containing 0–50 μM isoflavones, 10 mM buffer and 0–50 μM Aβs were analyzed at 25 °C with a black microfluorimeter cell (GL Sciences Inc., Tokyo, Japan). ΔFluorescence emission of Gly in the presence of various Aβ species was obtained after subtraction of both the fluorescence emission of Gly alone and that of Aβs alone from the actual fluorescence emission of Gly mixed with Aβs. The buffers used were citrate buffer at pH 4–6, phosphate buffer at pH 6–8, Tris–HCl buffer at pH 8–9 and glycine–NaOH buffer at pH 8.5–10. Excitation and emission fluorescence spectra were obtained immediately after the reaction mixture was made. Excitation and emission were scanned in the range of 200–600 nm and 200–600 nm, respectively. The scanning speed was 2400 nm/min, and excitation and emission slits were set at 5 and 5 nm, respectively. Scans were completed in 5 min.

2.12. Statistical analysis

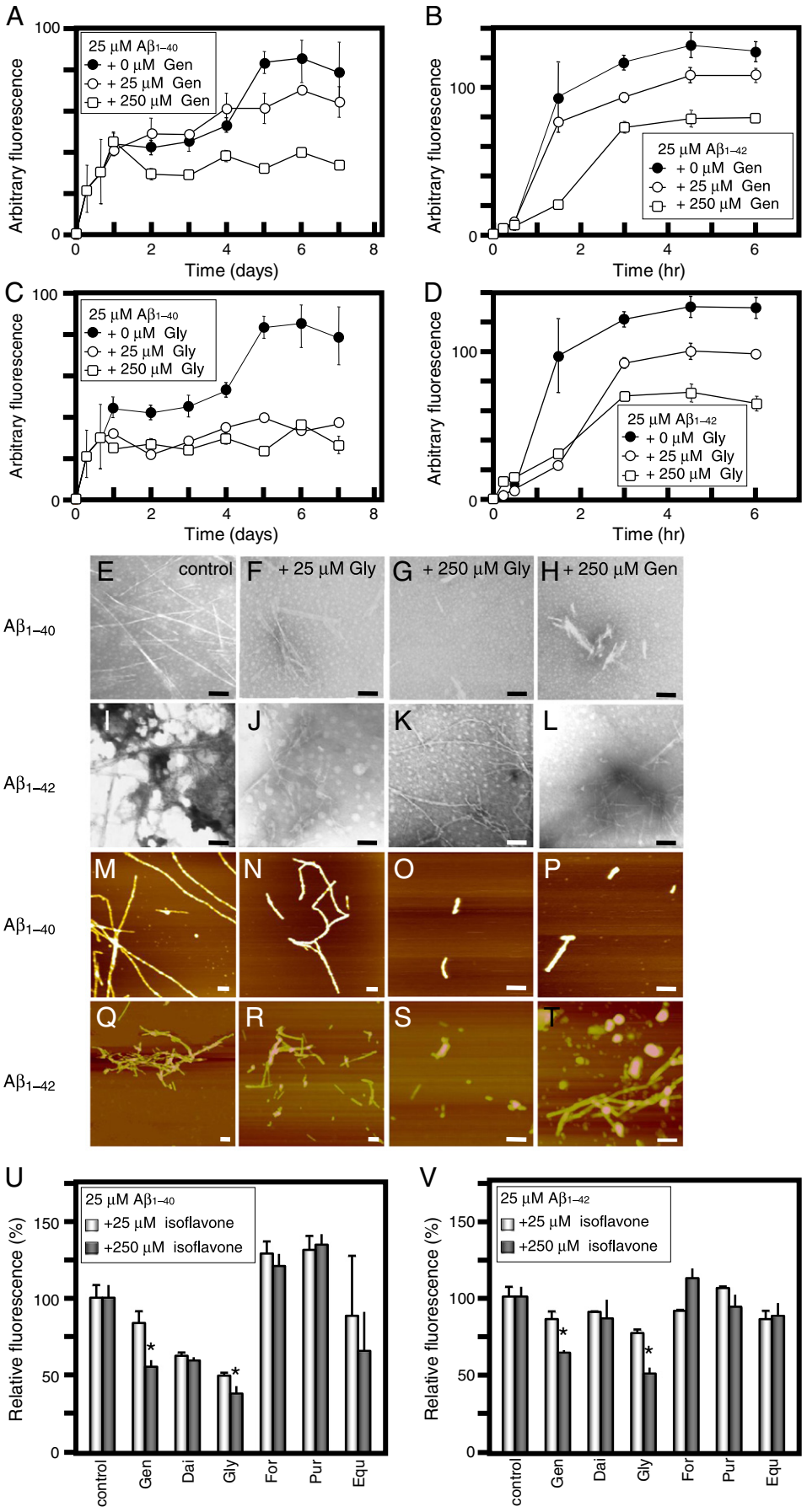
One-way factorial analysis of variance followed by Tukey–Kramer post-hoc comparisons were used to determine statistical significance among data sets. Significance was defined as $p<0.05$.

3. Results

3.1. Aβ fibrillization assay and anti-fibrillization effects of isoflavones on $A\beta_{1-40}$ and $A\beta_{1-42}$

When fresh $A\beta_{1-40}$ (Fig. 2A, C) or $A\beta_{1-42}$ (Fig. 2B, D) was incubated at 37 °C, the fluorescence gently increased, finally reaching a plateau. When $A\beta_{1-40}$ was incubated with 25 or 250 μM Gen (Fig. 2A) or Gly (Fig. 2C), the final equilibrium level decreased in a dose-dependent manner. Similar effects were observed for the two isoflavones with $A\beta_{1-42}$; Gen (Fig. 2B) and Gly (Fig. 2D). EM and AFM analyses revealed that the number of thick straight fibrils was reduced after incubating fresh $A\beta_{1-40}$ (Fig. 2F, N) or $A\beta_{1-42}$ (Fig. 2J,R) with 25 μM Gly. Short fibrils and amorphous aggregates were observed after incubating fresh $A\beta_{1-40}$ or $A\beta_{1-42}$ with 250 μM Gly (Fig. 2G,K,O,S) or Gen (Fig. 2H,L,P,T). Thus, Gen and Gly can inhibit the fibrillization of both $A\beta_{1-40}$ and $A\beta_{1-42}$. We compared the anti-fibrillization activities of the isoflavones (Fig. 2U,V); statistical analysis revealed a significant difference in the potency of 250 μM Gen and Gly on both $A\beta_{1-40}$ (Fig. 2U) and $A\beta_{1-42}$ fibrillization (Fig. 2V). Gly, at 250 μM, had the strongest activity.

Fig. 2. Anti-fibrillization effects of isoflavones on $A\beta_{1-40}$ and $A\beta_{1-42}$. (A–D) Effects of Gen (A, B) and Gly (C, D) on the kinetics of $fA\beta_{1-40}$ (A, C) and $fA\beta_{1-42}$ (B, D) formation from fresh $A\beta_{1-40}$ and $A\beta_{1-42}$, respectively, monitored by ThT fluorescence. The reaction mixtures, containing 25 μM $A\beta_{1-40}$ (A, C) or $A\beta_{1-42}$ (B, D), 10 mM phosphate buffer (pH 7.4), and 0 (●), 25 (○) or 250 μM (□) of Gen (A, B) or Gly (C, D), were incubated at 37 °C for the indicated times. Periodically, three 5-μL aliquots were removed, and ThT binding levels were determined. Binding is expressed as a mean fluorescence in arbitrary fluorescence units ± the standard error (SE) (error bars). Each figure comprises data obtained in 3 independent experiments. (E–T) Electron microscopy (E–L) and atomic force microscopy (M–T) of $A\beta_{1-40}$ and $A\beta_{1-42}$ assembly morphology. The reaction mixtures, containing 25 μM $A\beta_{1-40}$ (E–H, M–P) or $A\beta_{1-42}$ (I–L, Q–T), 10 mM phosphate buffer (pH 7.4), and 0 (E, I, M, Q), 25 (F, J, N, R) or 250 μM Gly (G, K, O, S), or 250 μM Gen (H, L, P, T) were incubated at 37 °C for 7 days (E–H, M–P) or 6 h (I–L, Q–T). Scale bars indicate 100 nm. (U, V) Effects of isoflavones on the formation of $A\beta_{1-40}$ fibrils ($fA\beta_{1-40}$) (U) and $fA\beta_{1-42}$ (V) from fresh $A\beta_{1-40}$ and $A\beta_{1-42}$, respectively, monitored by ThT fluorescence. The reaction mixture, containing 25 μM Aβ, 10 mM phosphate buffer (pH 7.4), and 25 (white columns) or 250 μM isoflavones (gray columns), was incubated at 37 °C for 7 days (U) or 6 h (V), respectively. Each column represents the average of 3 independent experiments. The average without compounds was regarded as 100%. Bars indicate SE. *$p<0.05$; post-hoc Tukey–Kramer tests.


A
25 μM Aβ1–40
+ 0 μM Gen
+ 25 μM Gen
+ 250 μM Gen
Arbitrary fluorescence
Time (days)
B
25 μM Aβ1–42
+ 0 μM Gen
+ 25 μM Gen
+ 250 μM Gen
Arbitrary fluorescence
Time (hr)
C
25 μM Aβ1–40
+ 0 μM Gly
+ 25 μM Gly
+ 250 μM Gly
Arbitrary fluorescence
Time (days)
D
25 μM Aβ1–42
+ 0 μM Gly
+ 25 μM Gly
+ 250 μM Gly
Arbitrary fluorescence
Time (hr)
E control
F + 25 μM Gly
G + 250 μM Gly
H + 250 μM Gen
Aβ1–40
I
J
K
L
Aβ1–42
M
N
O
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Aβ1–42
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25 μM Aβ1–40
+25 μM isoflavone
+250 μM isoflavone
Relative fluorescence (%)
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+250 μM isoflavone
Relative fluorescence (%)
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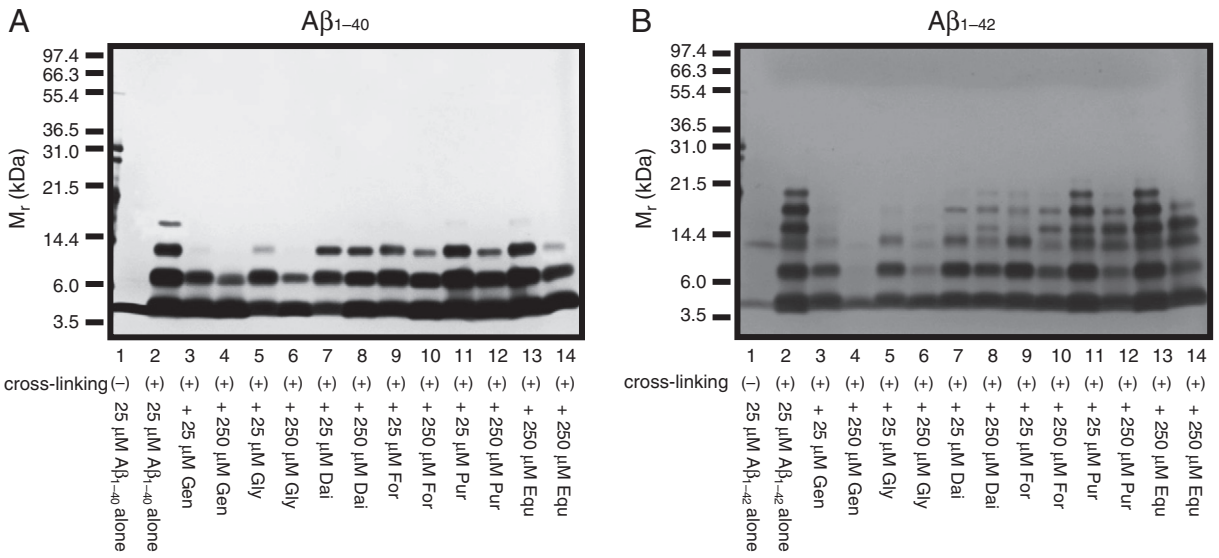


Fig. 3. Anti-oligomerization effects of isoflavones on $A\beta_{1-40}$ and $A\beta_{1-42}$. PICUP, followed by SDS-PAGE and silver staining, was used to determine the effects of isoflavones on $A\beta_{1-40}$ (A) and $A\beta_{1-42}$ oligomerization (B). Lanes 1, proteins alone (no cross-linking); lanes 2, proteins alone; lanes 3–14, proteins plus 25 or 250 μM isoflavones. Each gel is representative of each of 3 independent experiments.

3.2. Aβ oligomerization assay and anti-oligomerization effects of isoflavones on $A\beta_{1-40}$ and $A\beta_{1-42}$

We examined the effects of isoflavones on Aβ oligomerization by using PICUP, followed by SDS-PAGE and silver staining (Fig. 3). In the absence of cross-linking by PICUP as a control, only $A\beta_{1-40}$ monomers (Fig. 3A, lane 1) and $A\beta_{1-42}$ monomers and trimers (Fig. 3B, lane 1) were observed. The $A\beta_{1-42}$ trimer band has been shown to be an SDS-induced artifact [18]. After cross-linking, $A\beta_{1-40}$ existed as a mixture of monomers and oligomers of orders 2–4 (Fig. 3A, lane 2), whereas $A\beta_{1-42}$ comprised monomers and oligomers of orders 2–6 (Fig. 3B, lane 2). When 250 μM Gen or Gly was mixed with 25 μM $A\beta_{1-40}$, oligomerization was blocked significantly with only dimer bands visible (Fig. 3A, lanes 4 and 6). Mixing 25 μM Gen or Gly with 25 μM $A\beta_{1-40}$, produced lower levels of inhibition (Fig. 3A, lanes 3 and 5). The effects of Dai, For, Pur and Equ on $A\beta_{1-40}$ oligomerization were weaker compared with Gen or Gly, with low tetramer band intensities (Fig. 3A, lanes 7–14).

The effect of 250 μM Gen on $A\beta_{1-42}$ oligomerization was equally significant with very low intensities of oligomer bands (Fig. 3B, lanes 4 and 6). Mixing 25 μM Gen or Gly with 25 μM $A\beta_{1-42}$ produced weaker levels of inhibition (Fig. 3B, lanes 3 and 5). The effects of Dai, For, Pur and Equ on $A\beta_{1-42}$ oligomerization were weaker compared with Gen or Gly (Fig. 3B, lanes 7–14).

These data show that Gen and Gly inhibit $A\beta_{1-40}$ and $A\beta_{1-42}$ oligomerization at compound:peptide ratios of 1:10.

3.3. Aβ assembly-destabilizing assay and fibril-destabilizing effects of isoflavones on $A\beta_{1-40}$ and $A\beta_{1-42}$

After incubating fresh $fA\beta_{1-40}$ and $fA\beta_{1-42}$ at 37 °C without isoflavones as a control, ThT fluorescence did not change significantly (Fig. 4A, B). In contrast, when 250 μM Gly was incubated with 25 μM $fA\beta_{1-40}$, the final equilibrium level significantly decreased (Fig. 4A). Similar effects were observed when 250 μM Gen or Gly was incubated with 25 μM $fA\beta_{1-42}$ (Fig. 4B). EM analyses revealed that the number of fibrils was reduced slightly after incubating $fA\beta_{1-40}$ (Fig. 4D) or $fA\beta_{1-42}$ (Fig. 4H) with 25 μM Gly. Short aggregates and amorphous aggregates were observed after incubating $fA\beta_{1-40}$ or $fA\beta_{1-42}$ with 250 μM Gly (Fig. 4E, I) or Gen (Fig. 4F, J). AFM analyses revealed tangled and coated fibrils with amorphous aggregates after incubating $fA\beta_{1-40}$ or $fA\beta_{1-42}$ with Gly (Fig. 4L, M, P, Q) or Gen (Fig. 4N, R).

Fig. 4S and T shows the effects of Gly and Gen on preformed $fA\beta_{1-40}$ (Fig. 4S) or $fA\beta_{1-42}$ (Fig. 4T), determined by SDS-PAGE after fractionation into PB-sol (supernatant) and PB-insol (pellet) fractions. No proteins were detected in all of the PB-sol fractions. This implies that although Gly and Gen could destabilize $fA\beta_{1-40}$ and $fA\beta_{1-42}$ to visible aggregates (Fig. 4A–R) they could not depolymerize fAβs to PB-soluble Aβs including Aβ monomers.

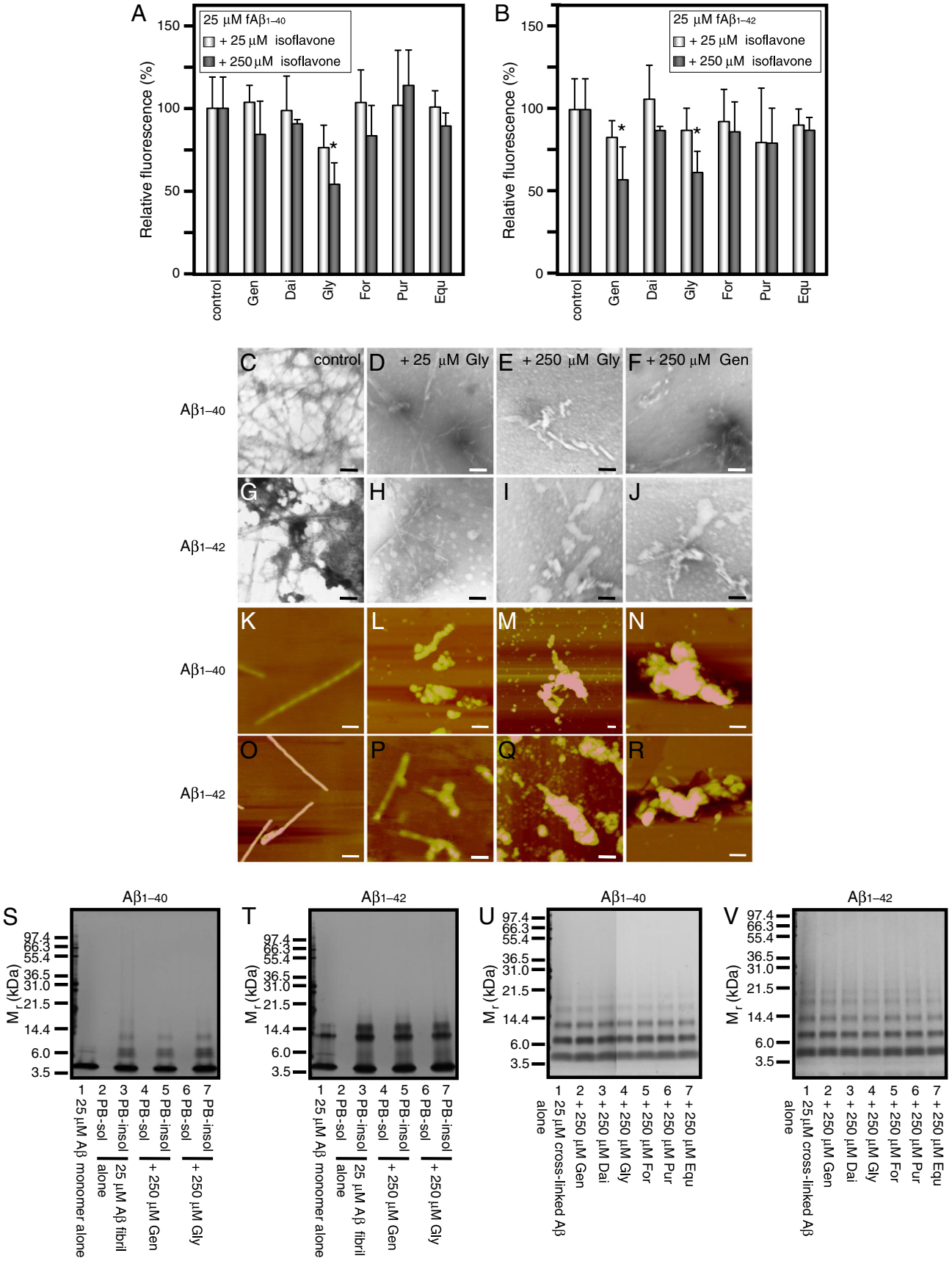
Fig. 4U and V shows the effects of 250 μM isoflavones on 25 μM cross-linked $A\beta_{1-40}$ (Fig. 4U) or $A\beta_{1-42}$ oligomers (Fig. 4V) determined by SDS-PAGE and silver staining. No isoflavone could change the distributions of oligomer frequency.

3.4. Characterization of the fluorescence emission of isoflavones in the presence or absence of various Aβ species

Solution of 12.5 μM Gly alone gave a maximum fluorescence emission (565 arbitrary units (AU)) at 465 nm with excitation at 350 nm (Supplemental Fig. S2; Gly, left). PB (control), Gen or Pur did not show obvious peaks of fluorescence emission (Supplemental Fig. S2; left). We confirmed that the peak fluorescence of isoflavones did not change for at least 5 min after the reaction mixture was made.

Fig. 4. Aβ assembly-destabilizing assay and fibril-destabilizing effects of isoflavones on $A\beta_{1-40}$ and $A\beta_{1-42}$. (A, B) The reaction mixture, containing 25 μM $fA\beta_{1-40}$ or $fA\beta_{1-42}$, 10 mM phosphate buffer (pH 7.4), and 25 (white columns) or 250 μM isoflavones (gray columns) was incubated at 37 °C for 24 h. Each column represents the average of 3 independent experiments. The average without compounds was regarded as 100%. Bars indicate SE. *$p<0.05$; post-hoc Tukey–Kramer tests. (C–R) Electron microscopic (C–J) and atomic force microscopy (K–R) analyses of $A\beta_{1-40}$ and $A\beta_{1-42}$ assembly morphology. The reaction mixtures, containing 25 μM $A\beta_{1-40}$ (C–F, K–N) or $A\beta_{1-42}$ (G–J, O–R), 10 mM phosphate buffer (pH 7.4), and 0 (C, G, K, O), 25 (D, H, L, P) or 250 μM Gly (E, I, M, Q), or 250 μM Gen (F, J, N, R) were incubated at 37 °C for 7 days (C–F, K–N) or 24 h (G–J, O–R). Scale bars indicate 100 nm. (S,T) Effects of Gly and Gen on fAβs-destabilization monitored by SDS-PAGE and silver staining. Lane 1, 25 μM $A\beta_{1-40}$ (S) or $A\beta_{1-42}$ monomer (T) alone; lanes 2–7, the reaction mixture containing 25 μM $fA\beta_{1-40}$ (S) or $fA\beta_{1-42}$ (T), 10 mM phosphate buffer (pH 7.4), and 0 or 250 μM fresh Gly or Gen was incubated at 37 °C for 24 h, fractionated into PB-sol and PB-insol fractions. Each gel is representative of each of 3 independent experiments. (U,V) Effects of isoflavones on the frequency of cross-linked Aβ oligomers monitored by SDS-PAGE and silver staining. The reaction mixture containing 25 μM cross-linked $A\beta_{1-40}$ (U) or $A\beta_{1-42}$ (V), 10 mM phosphate buffer (pH 7.4), and 0 or 250 μM fresh isoflavones was incubated at 37 °C for 24 h. Each gel is representative of each of 3 independent experiments.

The higher concentration of Gly was associated with a higher fluorescence intensity (Supplemental Fig. S3). The fluorescence change was linear from 0 to 25 μM Gly ($R^2 = 0.976$; Supplemental Fig. S3C), indicating that the fluorescence emitted at 465 nm with an excitation maximum at 350 nm reflects the specific fluorescence of the Gly solution.



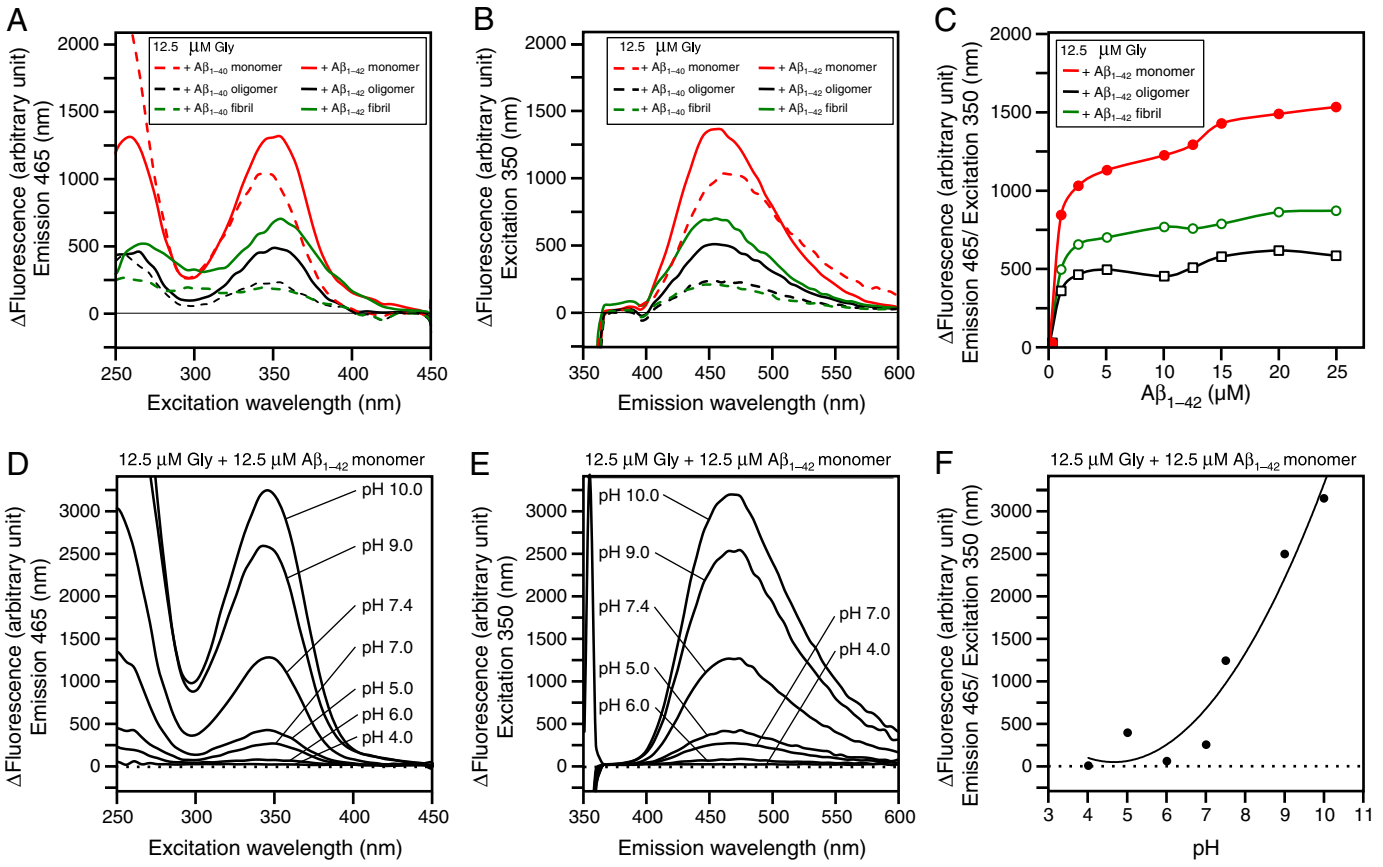


Fig. 5. ΔFluorescence emission of Gly in the presence of various Aβ species. ΔFluorescence emission of Gly in the presence of various Aβ species was obtained after subtraction of both the fluorescence emission of Gly alone and that of Aβs alone from the actual fluorescence emission of Gly mixed with Aβs. The data are representative pattern from 3 independent experiments. (A, B) ΔFluorescence spectra of Gly in the presence of various Aβ species. The reaction mixtures containing 0 or 12.5 μM $Aβ_{1–40}$ or $Aβ_{1–42}$ (monomers, fibrils or oligomers), 10 mM phosphate buffer (pH 7.4), and 0 or 12.5 μM Gly were monitored at an emission of 465 nm (A) or an excitation of 350 nm (B). (C) Effects of Aβ concentration on the Δfluorescence emission of Gly in the presence of Aβs. The reaction mixtures containing 0–25 μM $Aβ_{1–42}$ (monomers, fibrils or oligomers), 10 mM phosphate buffer (pH 7.4), and 12.5 μM Gly were monitored at the excitation and emission wavelengths of 350 and 465 nm, respectively. (D, E) ΔFluorescence spectra of Gly in the presence of $Aβ_{1–42}$ monomers. The reaction mixtures containing 0 or 12.5 μM $Aβ_{1–42}$ monomers, 10 mM buffer and 0 or 12.5 μM Gly were monitored at an emission of 465 nm (D) or an excitation of 350 nm (E). The buffers used were citrate buffer at pH 4–6, phosphate buffer at pH 6–8, Tris–HCl buffer at pH 8–9 and glycine–NaOH buffer at pH 8.5–10. (F) Effects of pH on the Δfluorescence emission of Gly in the presence of $Aβ_{1–42}$ monomers. The reaction mixtures containing 12.5 μM $Aβ_{1–42}$ monomers, 10 mM phosphate buffer (pH 4–10), and 0 or 12.5 μM Gly were monitored at the excitation and emission wavelengths of 350 and 465 nm, respectively.

Next, to examine whether Gly could interact with Aβ species, we used monomers, oligomers and fibrils of $Aβ_{1–40}$ or $Aβ_{1–42}$. Fluorescence emission of 12.5 μM $Aβ_{1–42}$ monomers or oligomers alone, obtained at the excitation and emission wavelengths of 350 and 465 nm, respectively, was neglectable (Supplemental Fig. S2 and S4). Solution of 12.5 μM $Aβ_{1–42}$ fibrils gave a maximum fluorescence emission of 410 nm with excitation at 300 nm (Supplemental Fig. S2), however, they gave little fluorescence emission at 465 nm with excitation at 350 nm (Supplemental Fig. S2 and S4). After mixing with $Aβ_{1–42}$ monomers, oligomers or fibrils, 12.5 μM Gly solution emitted a significantly higher fluorescence at 465 nm with an excitation maximum at 350 nm ($p<0.05$; Supplemental Fig. S2; Gly). Fig. 5 show the Δfluorescence excitation (Fig. 5A) and emission spectra (Fig. 5B) of Gly in the presence of individual Aβ species after subtracting each fluorescence of Gly and Aβ alone. The maxima of Δfluorescence emission were also observed at the excitation and emission wavelengths of 350 (Fig. 5A) and 465 nm (Fig. 5B), respectively. The higher concentration of $Aβ_{1–42}$ species was associated with a higher fluorescence intensity (Fig. 5C). The Δfluorescence of Gly in the presence of $Aβ_{1–42}$ monomer was pH-dependent, exhibiting a maximum around pH 10 and a minimum around pH 4–6 (Fig. 5D–F). These data indicates that the Δfluorescence obtained at an emission of 465 nm with excitation at 350 nm reflects the binding of Gly to Aβ monomers, oligomers and fibrils at pH 7.4.

After mixing $Aβ_{1–40}$ species with Gly, the Δfluorescence spectra were nearly identical to those of $Aβ_{1–42}$ species, and slightly lower fluorescence levels were observed compared to mixing $Aβ_{1–42}$ species with Gly (Fig. 5A,B). These data are indicating that Gly could interact with monomers, oligomers and fibrils of $Aβ_{1–40}$ and $Aβ_{1–42}$ in sample solution. In contrast, minimal changes in the fluorescence spectra were observed when $Aβ_{1–42}$ were mixed with PB (control), Gen, Dai, For, Pur or Equ (Supplemental Fig. S2).

3.5. The Δfluorescence emission of glycitein in the presence of Aβ fragments

To determine which regions of monomeric Aβs Gly interacts with, we used different Aβ fragments. Fig. 6 shows the Δfluorescence enhancement of Gly in the presence of Aβ fragments. The spectra of Gly mixed with $Aβ_{1–40}$ or $Aβ_{1–16}$ were nearly identical to that with $Aβ_{1–42}$. In comparison, a much larger Δfluorescence was observed after mixing Gly with $Aβ_{25–35}$ ($p<0.05$). Gly interacted with different Aβ fragments ($Aβ_{1–42}$, $Aβ_{1–40}$, $Aβ_{1–16}$ and $Aβ_{25–35}$), exhibiting the highest Δfluorescence enhancement with $Aβ_{25–35}$. Fig. 6C shows that the Δfluorescence intensity rose with increasing concentration of Aβ fragments. The Δfluorescence of Gly ultimately reached plateau levels at a compound:peptide ratio of 5:2 for $Aβ_{1–42}$, $Aβ_{1–40}$ and $Aβ_{1–16}$, and at a ratio of 1:1 or more for $Aβ_{25–35}$. These results suggest that Gly can

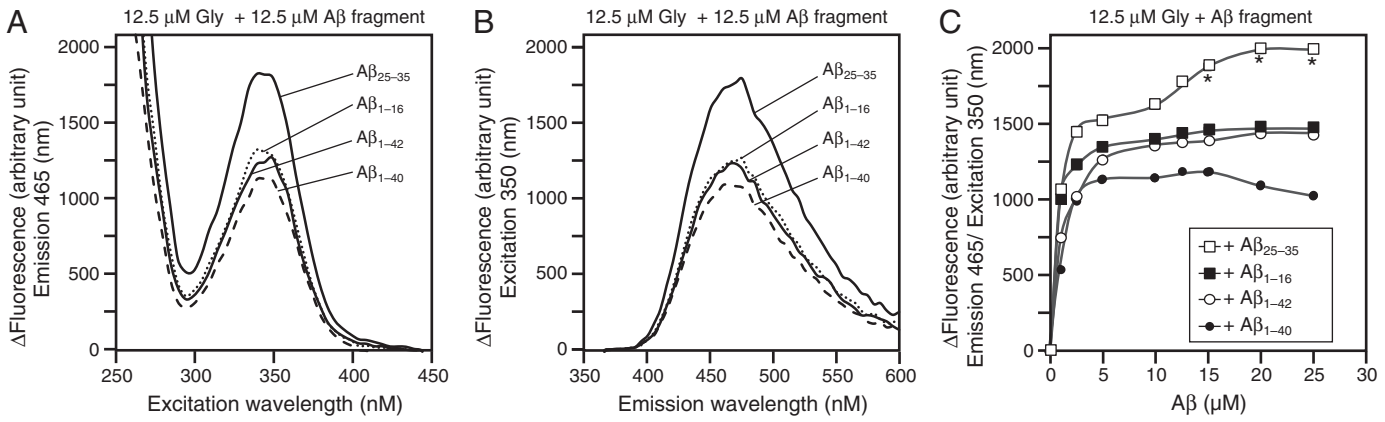


Fig. 6. ΔFluorescence emission of Gly in the presence of Aβ fragments. ΔFluorescence emission of Gly in the presence of Aβ fragments was obtained after subtraction of both the fluorescence emission of Gly alone and that of Aβ fragments alone from the actual fluorescence emission of Gly mixed with Aβ fragments. The data are representative pattern from 3 independent experiments. (A, B) ΔFluorescence spectra of Gly in the presence of Aβ fragments. The reaction mixtures containing 0 or 12.5 μM Aβ fragments ($Aβ_{1–16}$, $Aβ_{1–40}$, $Aβ_{1–42}$, $Aβ_{25–35}$), 10 mM phosphate buffer (pH 7.4), and 0 or 12.5 μM Gly were monitored at an emission of 465 nm (A) or an excitation of 350 nm (B). (C) Effects of Aβ concentration on the Δfluorescence emission of Gly in the presence of Aβ fragments. The reaction mixtures containing 0–25 μM Aβ fragments ($Aβ_{1–16}$, $Aβ_{1–40}$, $Aβ_{1–42}$, $Aβ_{25–35}$), 10 mM phosphate buffer (pH 7.4), and 0 or 12.5 μM Gly were monitored at the excitation and emission wavelengths of 350 and 465 nm, respectively. *$p<0.05$; post-hoc Tukey–Kramer tests.

bind to the limited number of interaction sites on $Aβ_{1–42}$, $Aβ_{1–40}$, $Aβ_{1–16}$ and $Aβ_{25–35}$, and that Gly may have the most potent affinity for $Aβ_{25–35}$ among the Aβ fragments examined in this study.

4. Discussion

We demonstrate that isoflavones, especially Gly and Gen, can inhibit the fibrillization as well as the oligomerization in a dose-dependent manner. Also, these compounds destabilized preformed $fAβ_{1–40}$ and $fAβ_{1–42}$. In addition, our three-dimensional fluorescence spectroscopic analyses showed that Gly directly interacted with monomers, oligomers and fibrils of $Aβ_{1–40}$ and $Aβ_{1–42}$ at pH 7.4, indicating the binding of Gly to these Aβ species. Moreover, Gly interacted with different Aβ fragments ($Aβ_{1–42}$, $Aβ_{1–40}$, $Aβ_{1–16}$ and $Aβ_{25–35}$), exhibiting the most potent affinity for $Aβ_{25–35}$. Gly can interact with the limited number of binding sites along the length of monomeric Aβs.

These interactions of Gly with Aβs would explain the anti-fibrillation and anti-oligomerization effects of Gly on $Aβ_{1–40}$ and $Aβ_{1–42}$. Gly initially binds with the site(s) present in monomeric Aβs, thereby preventing fibrillation and oligomerization. Alternatively, Gly binds to Aβ fibrils or oligomers, thus inhibiting the addition of Aβ monomers onto the growing ends of Aβ assembly. Previously, we demonstrated that a flavonoid myricetin bound to fAβs by using three-dimensional fluorescence spectroscopy and surface plasmon resonance analyses [16]. Prior work using circular dichroism (CD) spectroscopy and PICUP showed that a flavonoid could inhibit transitions of initial coil-α-helix/β-sheet secondary structure and subsequent pre-protofibrillar oligomerization [10].

The binding of Gly to fAβs would achieve the fibril-destabilizing effects of Gly on preformed $fAβ_{1–40}$ and $fAβ_{1–42}$. The fluorescence changes of $fAβ_{1–42}$ by Gly were slightly greater than those of $fAβ_{1–40}$, and, probably related to it, the fibril-destabilizing activities of Gly on $fAβ_{1–42}$ were slightly stronger than those on $fAβ_{1–40}$; this may be related to structural differences between $fAβ_{1–40}$ and $fAβ_{1–42}$. AFM analyses revealed fAβs coated and tangled into amorphous aggregates after the fAβ-destabilizing assay with Gly. By SDS-PAGE analyses after fractionation into PB-sol and PB-insol, Gly seems not to depolymerize fAβs to PB-soluble Aβs including Aβ monomers.

Although the fluorescence changes suggested the binding of Gly to Aβ oligomers, Gly could not destabilize preformed Aβ oligomers assembled by PICUP. There has been no report of compounds which destabilize preformed Aβ oligomers assembled by PICUP.

Among prior works on isoflavones, Gen has been reported to bind serum albumin noncovalently and exhibit enhanced fluorescence emission [21]; Gly, Gen and Dai have also been reported to bind the monomeric form of transthyretin protein (TTR) [22]. Because Gen did not show obvious peaks of fluorescence emission, our three-dimensional fluorescence spectroscopic methods were not available to analyze any interaction between Gen and Aβs, nor to explain the mechanism of Gen's anti-fibrillation, anti-oligomerization and fibril-destabilizing effects on Aβs.

Understanding of the relationship between the chemical structure of the flavonoids and these specific anti-amyloid effects may lead to development of more effective drug designs with more potent anti-amyloid effects, requiring further studies.

The anti-fibrillization, anti-oligomerization and fibril-destabilizing effects of isoflavones require concentrations of at least 250 μM in our *in vitro* system. Generally, under habitual dietary conditions, plasma Gly levels are in the range of 0.004–1.2 μM [23–25]. Six hours after intake of soymilk and tofu, 1.7 μM and 1.3 μM of Gen were measured in plasma, respectively [26]. Isoflavone concentrations of 0.5–0.9 μM were measured in the blood of 3 adults after consuming soy nutritional supplements [25]. Seven infants eating soy-based formula had 2–7 μM concentrations of isoflavones in their blood [25]. Although it was reported that the penetration of flavonoids into the central nervous system depends on their lipophilicity [27], there is little information about the interaction of isoflavones with the blood–brain barrier. In any case, it is unlikely that the estimated isoflavone concentrations in the cerebrospinal fluid (CSF) would be higher than 10 μM under habitual dietary conditions, and these would be lower than those used in this study. However, low concentrations of isoflavones may exhibit anti-amyloidogenic activities *in vivo* when administered for a long period. Alternatively, supplemental intake of isoflavones could sufficiently increase CSF concentrations to provide anti-amyloidogenic effects. There have been no clinical trials on the neurological effects of isoflavone consumption. The central nervous system levels of isoflavones have never reported in the studies using animal model fed with isoflavones. Previous reports demonstrated that long-term orally administered different polyphenolic flavonoids, including (+)-catechin and (−)-epicatechin, significantly attenuated cognitive deterioration and improved cognitive function in a mouse model of AD with reduced oligomeric Aβ in the brain [28,29]; the calculated total metabolite level in the brain was at the very most 1.1 pM after treatment with orally 200 mg/kg/day of the polyphenolic flavonoids, equivalent to a human dosage of 1 g/day [29].

Recent laboratory findings indicate that isoflavones may protect against dementia. Gen was protective against oxidative injury caused by $A\beta_{25-35}$ treatment, and it helped to maintain redox balance in PC12 cells [30]. Gen could also rescue neurons from Aβ-induced cell death by inhibiting the activation of p38 MAP kinase [31]. Furthermore, Gen can inhibit Aβ–associated inflammation—this appeared to be due to its ability to induce PPAR-γ expression in cultured astrocytes [32]. Mixed soy isoflavones, which contain Gen, Dai and Gly, attenuate oxidative stress and improve parameters related to aging and Alzheimer's disease in C57BL/6J mice treated with D-galactose [33]. In transgenic *Caenorhabditis elegans*, Gly may protect against Aβ-induced toxicity through its combined ability to mitigate oxidative injury and inhibit Aβ deposition [34]. Finally, this study demonstrates that isoflavones, especially Gly and Gen, can inhibit Aβ assembly, and that Gly's effects may be mediated by direct binding to Aβ monomers, oligomers and fibrils. Our results indicate that compounds with an isoflavone backbone may also be promising candidates for application in therapeutic strategies targeting Aβ assemblies.

In conclusion, we show that isoflavones, especially Gly and Gen, have anti-fibrillization, anti-oligomerization and fibril-destabilizing effects on $A\beta_{1-40}$ and $A\beta_{1-42}$ *in vitro*. In particular, Gly exerted an anti-amyloidogenic effect by binding to Aβ monomers, oligomers and fibrils. Gly interacted with the limited number of binding sites along the length of monomeric Aβ, and with different Aβ fragments ($A\beta_{1-42}$, $A\beta_{1-40}$, $A\beta_{1-16}$ and $A\beta_{25-35}$) exhibiting the most potent affinity for $A\beta_{25-35}$. Isoflavones may hold promise as a treatment option for preventative strategies targeting amyloid formation in Alzheimer's disease.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2012.05.006.

Acknowledgements

This work was supported in part by a grant from the Knowledge Cluster Initiative [High-Tech Sensing and Knowledge Handling Technology (Brain Technology)] (M.Y.); Grant-in-Aid for Scientific Research (B) (20390242) (M.Y.), and Grant-in-Aid for Young Scientists (B) (K.O.) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Japan; a grant for the Amyloidosis Research Committee from the Ministry of Health, Labour, and Welfare, Japan (M.Y.); a grant from the Novartis Foundation for Gerontological Research (K.O.); a grant from Nagao Memorial Foundation (K.O.); Alumni Association of the School of Medicine at Showa University (K.O.); and a grant from Fuji Foundation for Protein Research (M.H.).

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