The Inhibition of Amyloid Fibrillation Using the Proteolytic Products of PQQ-Modified α-Synuclein

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Abstract: The inhibition of amyloid fibril and/or oligomer formation allows a novel therapeutic approach to neurodegenerative diseases such as Parkinson's disease. We have previously reported that pyrroloquinoline quinone (PQQ), a cofactor in the bacterial oxidative metabolism of alcohols, prevents the amyloid formation of α-synuclein, which is the causative factor of Parkinson's disease. Moreover, PQQ-modified α-synuclein is also able to inhibit the fibrillation of intact α-synuclein. Here, we demonstrate that PQQ-modified peptide fragments, the proteolytic products of PQQ-modified α-synuclein, prevent the amyloid formation of full-length α-synuclein, and that these inhibitory effects are derived from the PQQ modification of the peptide. Moreover, these effects are likely to be peptide-sequence-dependent. Thus, the specific interaction between the full-length α-synuclein and the peptide region of the PQQ-modified peptide prevents amyloid formation.

Key Words: α-Synuclein, amyloid fibril, inhibitor, Parkinson's disease, peptide, pyrroloquinoline quinone.

INTRODUCTION

Conformational diseases, represented by neurodegenerative diseases, are characterized by the formation and accumulation of misfolded proteins or amyloid fibrils. Considering that the formation of oligomers and/or amyloid fibrils plays a crucial role in the toxicity of the proteins and the onset of consequent neurodegenerative diseases, the inhibition of their formation allows a novel therapeutic approach [1-4].

Several small compounds have been reported to prevent the fibril formation of amyloid-forming proteins [5-11]. Some of these compounds have a quinone structure, and covalently bind to the Lys residues of the amyloid-forming protein *via* Schiff-base formation [8-11].

We have also demonstrated that pyrroloquinoline quinone (PQQ) (Fig. **1A**), which is a cofactor in the bacterial oxidative metabolism of alcohols, prevents the amyloid formation of α-synuclein (α-Syn), which plays a crucial role in the onset of Parkinson's disease [12]. We have concluded that PQQ inhibits α-Syn fibrillation by binding with α-Syn covalently *via* Schiff-base formation (Fig. **1B**). Moreover, PQQ-modified α-Syn is also able to prevent α-Syn fibril formation [12].

This inhibitory effect of PQQ-modified α-Syn is considered to be due to the intermolecular interaction between PQQ-modified α-Syn and intact α-Syn. However, it is still unclear whether or not PQQ-modified full-length α-Syn must interact with intact α-Syn to inhibit the consequent fibrillation. For therapeutic applications, a PQQ-modified peptide with a smaller molecular weight is likely to be more appropriate than the PQQ-modified full-length protein, which has a higher molecular weight (molecular weight of full-length α-Syn, 14400 Da).

In this paper, we investigate the effects of proteolytic α-Syn and PQQ-modified α-Syn on full-length α-Syn amyloid formation. We demonstrate that proteolytic products of PQQ-modified α-Syn inhibit the amyloid formation of full-length α-Syn. Our current results suggest that inhibitory effects of PQQ-modified peptides are likely due to the PQQ modification of specific sequences, as not all proteolytic product of PQQ-modified α-Syn showed the inhibition.

METHODS

Chemicals

Pyrroloquinoline quinone (PQQ) was kindly donated by Mitsubishi Gas Chemical Company, Inc.

Preparation of Recombinant α-Synuclein

Human wild-type α-Syn was expressed in the *E. coli* BL21 (DE3) cell line transfected with the pET28a(+)/α-Syn plasmid and purified, as we reported previously [12].

PQQ Modification of α-Synuclein

PQQ-modified α-Syn was prepared as we reported previously [12]. α-Syn (140 μM) and PQQ (1.4 mM) were co-incubated in PBS buffer (8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3) at 37°C with shaking for over 100 h. Then, the incubated sample was loaded onto a PD-10 column (GE Healthcare Bio-science

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Corp.) to remove the intact PQQ. The eluted fractions containing adduct without intact PQQ were collected as PQQ-modified α-Syn (α-Syn-PQQ).

Proteolytic Digestion of α-Synuclein and PQQ-Modified α-Synuclein

Limited proteolysis of α-Syn and α-Syn-PQQ with endoproteinase Glu-C was performed at 37°C for 18 h according to the manual described in the product information for Glu-C. The reaction was conducted at a mixture ratio of 3/100 (w/w) in PBS buffer, and the protein concentrations of α-Syn and α-Syn-PQQ were kept at 4 mg/ml. Under these conditions, Glu-C digests the carboxyl side of both the Asp and the Glu residues specifically (Fig. **1C**). After proteolytic digestion, the undigested α-Syn/α-Syn-PQQ and Glu-C were removed from the proteolytic mixtures by ultrafiltration through Amicon Ultra-4 filters (MWCO, 5000 Da; Millipore). The resulting ultrafiltrate containing digested α-Syn/α-Syn-PQQ was further separated by reverse-phase chromatography (RPC). The ultrafiltrate was then loaded onto a Resource RPC column (column volume, 1 ml; GE Healthcare Bio-science Corp.) and eluted with a 2-80% acetonitrile gradient in buffer containing 0.05% trifluoroacetic acid (TFA). The eluate was monitored by the absorbance at 210 nm (Abs_{210}) and 280 nm (Abs_{280}) simultaneously. Each 1-ml eluted fraction was collected and stored at -80°C after lyophilization. The proteolytic α-Syn fractions were designated as S-1, S-2, S-3, and so on, in the order of elution, and those of α-Syn-PQQ were correspondingly designated as SP-1, SP-2, SP-3, and so on.

Amyloid Fibril Formation Analysis

Purified α-Syn was ultracentrifuged (150000*g*, 1 h, 4°C) to remove any aggregates. The lyophilized proteolytic products of α-Syn and α-Syn-PQQ were dissolved in PBS buffer. Each dissolved proteolytic product was mixed with 1.0 mg/ml (70 μM) α-Syn. The concentration of each proteolytic product in the mixed solution was 3-fold higher than that of the eluate. A control solution containing 1.0 mg/ml (70 μM) of α-Syn alone was also prepared. All samples contained 0.02 % NaN_3 as an antiseptic agent. For each sample, 200 μl were aliquoted in triplicate into a 96-well microtiter plate

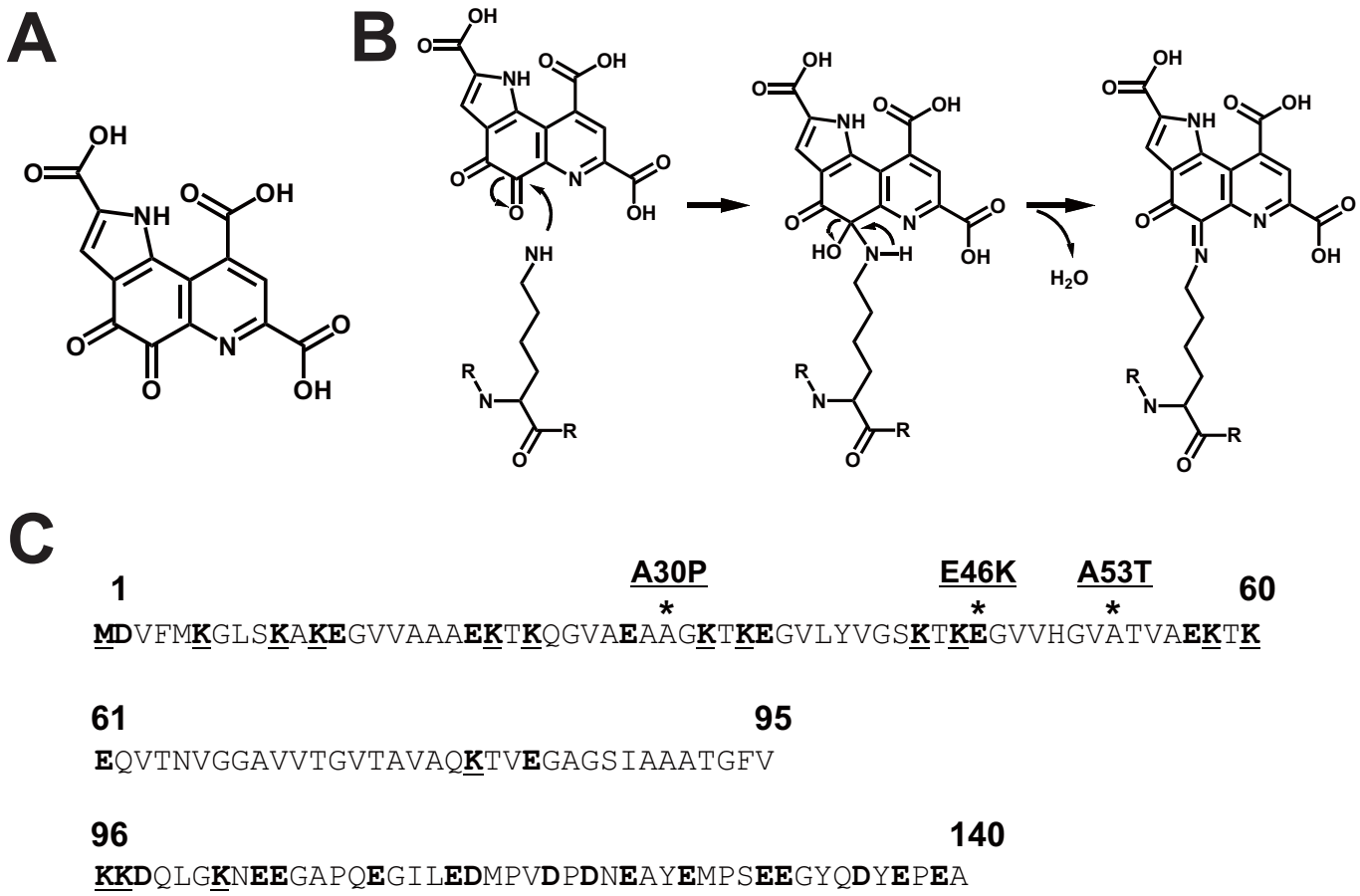

Fig. (1). Structure of pyrroloquinoline quinone (PQQ) (A), scheme of its Schiff-base formation with Lys residues of the peptides (B) and primary structure of α-synuclein (C). (**A**) Chemical structure of PQQ. (**B**) "R" indicates the amino-acid residue next to the Lys residue. (**C**) The primary structure of α-synuclein can be divided into three characteristic regions: the N-terminal region (1-60; top), the NAC region (61-95; middle) and the C-terminal region (96-140; bottom). The positions of three missense mutations (Ala53Thr, Ala30Pro and Glu46Lys) that are related to the early onset of Parkinson's disease are marked with asterisks (*). PQQ binds to the Lys (K) residues and amino group of the first Met (M) residue (bold and underlined) *via* Schiff-base formation. Endoproteinase Glu-C specifically digests the carboxyl side of the Asp (D) and the Glu (E) residues (bold).

together with a Teflon ball. The plates were covered with a seal and incubated at 37°C with shaking at approximately 700 rpm. Each sample was run in 3-5 replicates. Amyloid formation was monitored by thioflavin T (TfT) fluorescence. Aliquots of 2.5 μl were removed from the incubated sample and added to 250 μl of 25 μM TfT in PBS buffer. TfT fluorescence was recorded at 486 nm with excitation at 450 nm using an ARVO MX 1420 multilabel counter (PerkinElmer). The lag-time values were calculated by the sigmoidal curve fitting of the measured data using GraphPad Prism 4.00 (GraphPad Software, Inc.). In the TfT solution, the compounds examined in this study were diluted down to 1/100 of the concentration in the measurement mixture, which is enough to avoid the quenching effect of PQQ on the TfT analysis.

RESULTS AND DISCUSSION

Isolation of the Proteolytic Products of α-Synuclein and PQQ-Modified α-Synuclein

After Glu-C digestion, the proteolytic α-Syn and α-Syn-PQQ were separated by means of reverse-phase chromatography (RPC). The eluate was monitored by the absorbance at 210 nm (Abs_{210}) and 280 nm (Abs_{280}) simultaneously.

Figs. (**2A** and **2B** top) show the chromatograms for Glu-C-digested α-Syn and α-Syn-PQQ detected at Abs_{210}, respectively. Almost similar chromatograms were observed in these analyses, although there were differences in peak intensity. Three major peaks at a retention time of 5-8 min (Fig. **2**, top, peaks (i), (iii) and (iv)), and four minor peaks at a retention time of 8-13 min were observed in both chromatograms (Figs. **2A** and **2B**, top).

On the other hand, the chromatograms monitored at Abs_{280} showed significant differences (Figs. **2A** and **2B**, middle). The chromatogram for Glu-C-digested α-Syn monitored at Abs_{280} showed only major peaks at a retention time of 6-8 min (Fig. **2A**, middle, peaks (iii) and (iv)). Since aromatic amino acids have absorption at 280 nm, these peaks were probably derived from peptides containing aromatic residues such as Tyr, Phe and Pro (α-Syn has no Trp residue). However, the chromatogram for Glu-C-digested α-Syn-PQQ showed a different pattern (Fig. **2B**, middle). Two major peaks (Fig. **2B**, middle, peaks (ii) and (iv)) and two minor peaks (Fig. **2B**, middle, peaks (i) and (iii)) were observed at a retention time of 5-8 min. The latter two peaks (Fig. **2B**, middle, peaks (iii) and (iv)) were also observed in the chromatogram for Glu-C-digested α-Syn (Fig. **2A**, middle, peaks (iii) and (iv)). However, the earlier two peaks (Fig. **2B**, middle, peaks (i) and (ii)) were observed only in the chromatogram for Glu-C-digested α-Syn-PQQ (Fig. **2A**, middle). Furthermore, several additional broad peaks at a retention time of 13-15 min and 20-23 min were also observed, but only in the chromatogram for Glu-C-digested α-Syn-PQQ (Fig. **2B**, middle). In our previous study, PQQ-modified α-Syn was revealed to show characteristic adsorption in the vicinity of 280 nm, which was not observed in the intact α-Syn [12]. This adsorption is due to the chromogenic property of PQQ. Therefore, the observed peaks showing adsorption in the vicinity of 280 nm represent the PQQ-modified partial peptides caused by the Glu-C digestion of α-Syn-PQQ.

In order to investigate the inhibitory effect of PQQ-modified α-Syn partial sequences derived from Glu-C digestion on the amyloid formation of α-Syn, we recovered fractions from Glu-C-digested α-Syn-PQQ which showed adsorption in the vicinity of 280 nm, at a retention time of 5-15 and 20-23 min (SP represents the fraction from Glu-C digested α-Syn-PQQ; hereinafter, SP-6 to SP-15 and SP-21 to SP-23), and subjected them to further investigation. As controls, the fractions from Glu-C-digested α-Syn eluted for the same retention period as the above-mentioned Glu-C-digested α-Syn-PQQ fractions (S represents the fraction from Glu-C digested intact α-Syn; hereinafter, S-6 to S-15 and S-21 to S-23), were subjected to the same investigation.

The Inhibitory Effect of the Proteolytic Products of PQQ-Modified α-Syn Partial Sequences on the Amyloid Formation of α-Syn

The amyloid-fibril formation of full-length α-Syn was investigated in the presence or absence of Glu-C-digested α-Syn-PQQ fractions or Glu-C-digested α-Syn fractions. The amyloid-fibril formation in each investigation was monitored by the increase in the TfT fluorescence intensity at 486 nm with excitation at 450 nm. The results of the TfT analysis are shown in Figs. (**2** bottom and **3**). Fig. (**2** bottom) summarizes the TfT fluorescence intensity after 84 h of incubation for each sample, showing the quantity of amyloid fibrils formed relative to the sample in which full-length α-Syn was incubated without any proteolytic product. Amyloid firbril formation is considered to be nucleation-dependent, which is composed of nucleation, extension and equilibrium phases [13-15]. The final amount of amyloid fibril at certain incubation time represents fibrillation tendency including these three steps.

Among the samples tested (SP-6 to SP15 and SP-21 to SP-23), Glu-C-digested α-Syn-PQQ showing adsorption in the vicinity of 280 nm, SP-6, SP-7, SP-15 and SP-22, showed a marked inhibitory effect on the amyloid formation of full-length α-Syn, compared with the amyloid formation in the absence of any proteolytic products (Fig. **2B**, bottom). The amyloid formation of these samples were 44.1% (SP-6), 22.6% (SP-7), 26.8% (SP-15) and 27.6% (SP-22) of the amyloid formation observed in the absence of these fractions. However, most of the controls containing Glu-C-digested α-Syn fractions (S-6 to S-15 and S-21 to S-23) showed similar amounts of amyloid formation as the samples without proteolytic products (Fig. **2A**, bottom). S-15 showed an inhibitory effect, and amyloid formation was only 40% compared with the samples without the fraction, although the impact was lower than that of the corresponding Glu-C-digested α-Syn-PQQ fraction, SP-15 (26.8%). These results indicate that some of the proteolytic products of α-Syn-PQQ have the potential to inhibit the amyloid-fibril formation of full-length α-Syn.

For further consideration, we compared the time courses of amyloid formation monitored by the TfT fluorescence of the samples containing the Glu-C-digested α-Syn-PQQ fractions SP-7, SP-15 and SP-22, which showed significant inhibitory effects, and the corresponding Glu-C-digested α-Syn fractions S-7, S-15 and S-22 (Fig. **3**). The incubation of α-Syn in the absence of proteolytic products showed a gradual

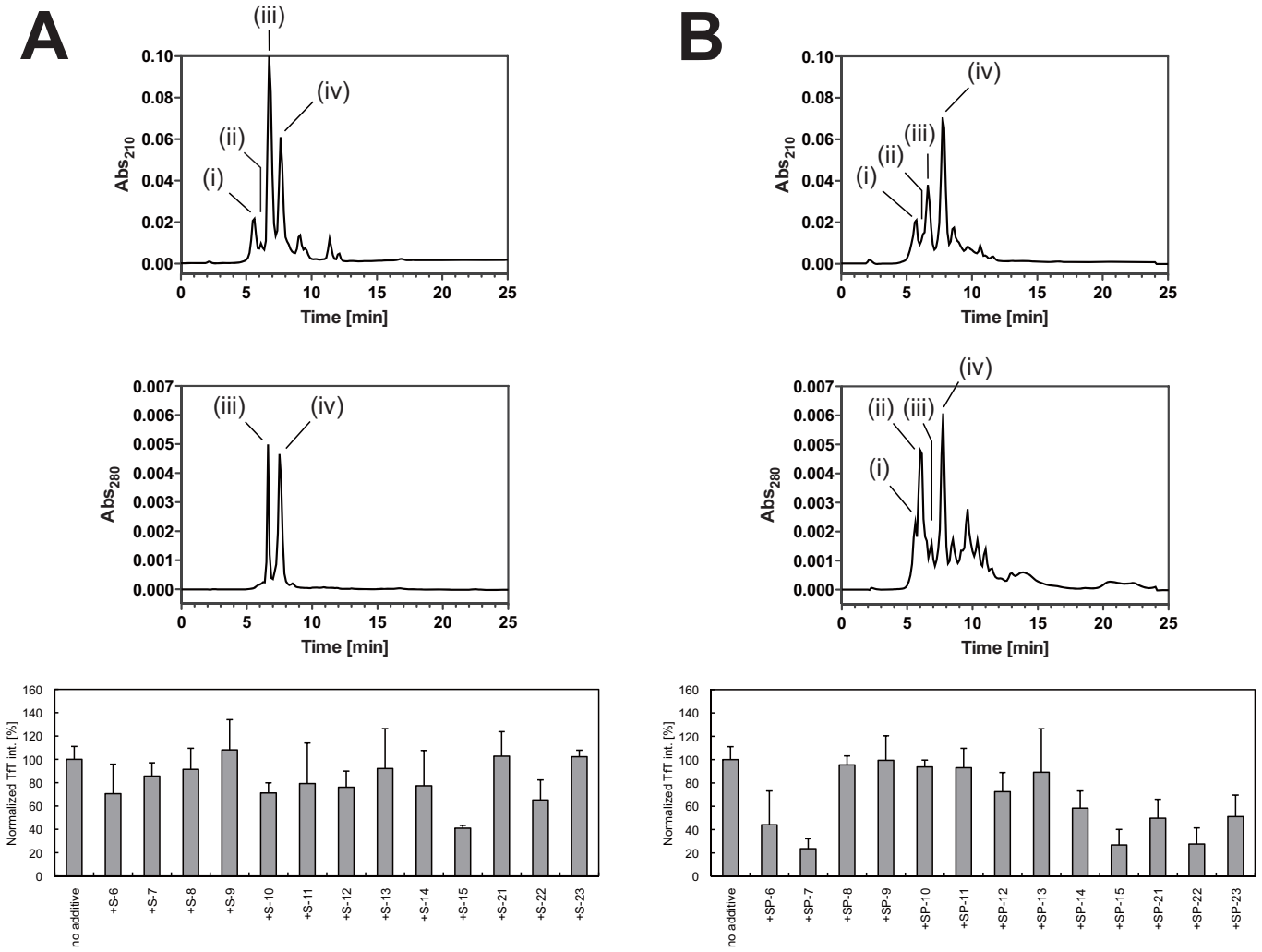

Fig. (2). Results of reverse-phase chromatography (RPC) separation of the proteolytic products of α-synuclein (α-Syn) and PQQ-modifeid α-synuclein (α-Syn-PQQ) and their effects on the amyloid fibril formation of full-length α-Syn. Glu-C-digested α-Syn (**A**) and α-Syn-PQQ (**B**) were separated by means of RPC. The eluate was detected at Abs_{210} (top) and Abs_{280} (middle) simultaneously. Roman numbers ((i) to (iv)) represent peaks at a retention time of 5-8 min and each number correspond to each peak observed at same retention time. After lyophilization, the effects of each fractionated Glu-C-digested α-Syn (**A**, bottom) and α-Syn-PQQ (**B**, bottom) on full-length α-Syn amyloid formation were investigated. The vertical and horizontal axes indicate the relative final TfT intensity and the added fraction, respectively. The results are expressed as percentages of the value of α-Syn alone ("no additive"), which is set at 100% *n=3*.

increase along a typical sigmoidal curve, with a lag time of 46.3 h (Fig. **3**, "No additive"), which is consistent with the typical amyloid-fibril formation kinetics as reported previously. S-7 did not show any significant inhibitory effect on full-length α-Syn fibrillation, although a prolonged lag time in the increasing TfT fluorescence was observed. In contrast, SP-7 showed a marked decrease in TfT fluorescence (Fig. **3A**). As shown in Figs. (**2A** and **2B** bottom), both SP-15 and S-15 showed a marked decrease in amyloid formation. However, the time courses of amyloid formation in these samples were different. SP-15 showed a decrease in amyloid formation and lag time is almost same as control, whereas S-15 showed a reduced lag time with decreased amyloid formation (lag time of the control, 46.3 h; S-15, 4.1 h; SP-15, 45.7 h) (Fig. **3B**). As described above, amyloid fibrillation is considered to be nucleation-dependent, which is composed of three steps; nucleation, elongation and equilibrium phase. Lag time represents the tendency of fibril nucleus formation, which is first step of amyloid fibrillation [13-15]. These results indicate that S-15 facilitates nucleation step of full-length α-Syn, while SP-15 dose not. SP-22 showed decreased amyloid formation, and S-22 also showed decreased amyloid formation, but the impact was smaller than in SP-22 for a similar time course (Fig. **3C**).

Assuming that the fractions with corresponding numbers (corresponding proteolytic products) contain corresponding regions of the peptide fragments, these results indicate that the inhibitory effect of the PQQ-modified peptide fragments is due to the PQQ modification of the peptide fragments (Fig. **3**). As not all PQQ-modified peptides inhibit full-length α-Syn fibrillation (Fig. **2**, bottom), this inhibitory effect is likely to be peptide-sequence-dependent.

Our results suggest that PQQ-modified peptide fragments recognize and interact with the full-length protein and consequently inhibit fibrillation. Therefore, the peptide domain of the PQQ-modified peptide would recognize and interact with the full-length α-Syn protein. Indeed, several peptide inhibitors have been reported and are expected to recognize target proteins specifically [16]. Moreover, the non-specific interaction of PQQ would also be avoided. Due to its high reactivity, PQQ is likely to react easily with the amino group of proteins or amino acids, consequently binding *via* Schiff-base formation. Forming an adduct with the peptide, the quinone domain of PQQ is blocked by Schiff-base formation (Fig. **1B**), and thus any undesirable non-specific interactions would be avoided. Therefore, the PQQ modification of the peptide would confer specificity to PQQ.

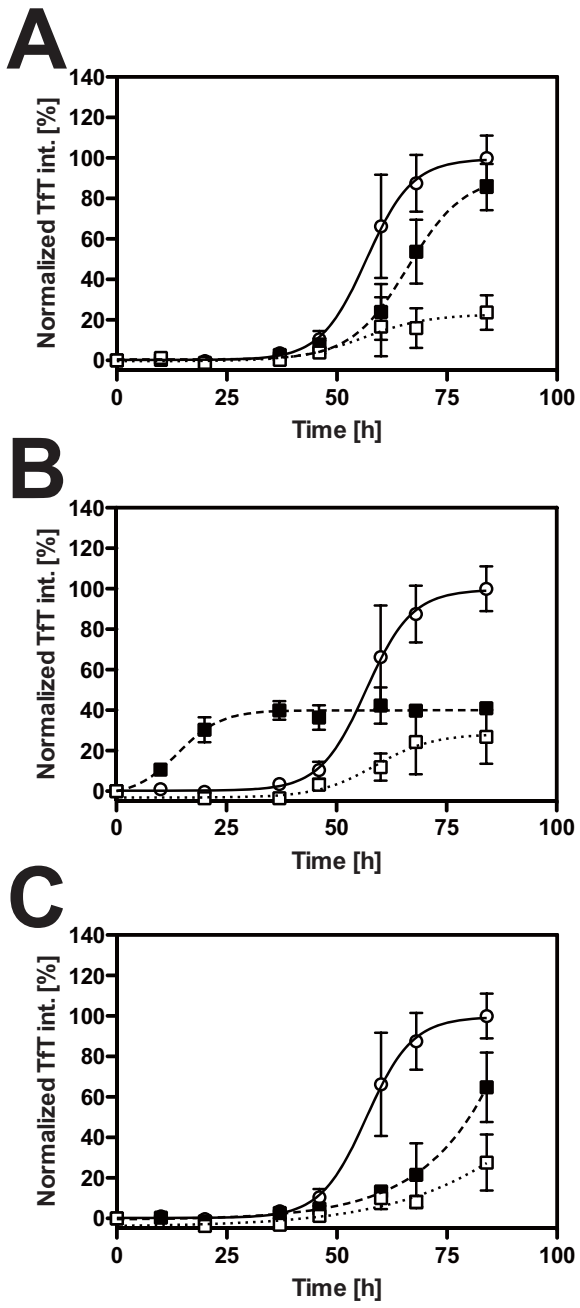

Fig. (3). Comparison of the effects of the fractions of proteolytic α-synuclein (α-Syn) and PQQ-modified α-synuclein (α-Syn-PQQ) with corresponding numbers on full-length α-synuclein amyloid formation. The time course of α-synuclein fibrillation and its mixtures with fractions 7 (**A**), 15 (**B**) and 22 (**C**). The final TfT intensity of the control sample, which contains α-synuclein alone, is set at 100%: no additive (*white circles*), + proteolytic α-Syn (*black squares*), + proteolytic α-Syn-PQQ (*white squares*) *n=3*.

The strategy of modifying a partial peptide fragment with a small compound to confer specificity to a small-molecule inhibitor can be applied to other amyloid-forming proteins utilizing the corresponding partial peptide fragments. Furthermore, it is also applicable to other small molecules that inhibit fibrillation by binding to the amyloid-forming protein covalently (such as dopamine, L-dopa, baicalein or rifampicin) [8-11]. Thus, this current strategy can confer specificity to inhibitors against various kinds of amyloid-forming proteins.

Although peptide-sequence identification is needed for further applications, this current basic strategy can be used to construct target-specific amyloid inhibitors, which may lead to the development of novel therapeutic approaches to conformational diseases.

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