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Construction of a chemically and conformationally self-replicating system of amyloid-like fibrils

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Abstract—The amyloid-like fibril is considered to be a macromolecular self-assemblage with a highly-ordered quaternary structure, in which numerous β-stranded polypeptide chains align regularly. Therefore, this kind of fibril has the potential to be engineered into proteinaceous materials, although conformational alteration of proteins from their native form to the amyloid form is a misfolding and undesirable process related to amyloid diseases. In this study, we have attempted to design an artificial system to explore applicability of using the amyloid-like fibril as a construct possessing self-recognition and self-catalytic abilities. A peptide self-replicating system based on the β-structure of the amyloid-like fibril was designed and constructed. The β-stranded peptide was self-replicated by the native chemical ligation reaction, and the newly generated peptide was self-assembled into amyloid-like fibrils. Thus, the constructed system was of both chemical and conformational self-replicating fibrils.

1. Introduction

Conformational alteration of proteins from their native form to the amyloid form is a misfolding and undesirable process, since the amyloid fibril has been proposed to be a causative agent for a variety of fatal diseases known as amyloid diseases, such as Alzheimer's and prion diseases.[1–4] A model system using simplified designed peptides may lead to a better understanding of underlying mechanisms by which polypeptides assemble into the fibrils, and potentially to the development of therapies against amyloid diseases.[1] On the other hand, the amyloid fibril is considered to be a macromolecular self-assemblage with a highly-ordered quaternary structure, in which numerous β-stranded polypeptide chains align regularly,[2,5] and thus this kind of fibril has the potential to be engineered into proteinaceous materials.[6–9] The design strategy to construct amyloid-like fibrils composed of peptides with a simplified primary structure, such as that reported previously,[1] will be advantageous toward engineering applications of this macromolecular assemblage. Especially, the method to construct amyloid-like fibrils composed of heterogeneous multiple peptide species[1e,f] will make the construct further applicable to bionanotechnology researches, because the alignment of these species can be predetermined, and thus the site-specific incorporation of multiple kinds of functional groups and their sequential array will be possible.

One reason why this construct is promising for material use is that the amyloid fibril intrinsically possesses an ability of molecular recognition. It has been reported that several kinds of molecules (e.g., small organic compounds,[10–12] proteins[13] and sugars[14]) bind specifically to the amyloid fibrils of proteins but not to non-amyloid form with the identical chemical structure. The amyloid fibril can act as a construct of macromolecular host to recognize specific molecules and capture them. The molecular recognition and binding exhibited by the amyloid fibril might occur on its highly organized chiral surface, which is provided by well-arranged polypeptide side chains and backbones. The primary molecular recognition of the amyloid fibril is the self-recognition, because, in general, the amyloid fibril is a macromolecular self-assemblage composed of single polypeptide species. The amyloid fibril recognizes polypeptide chains possessing the identical chemical structure. The recognized peptides are assembled on the fibril, in which the conformation of the newly assembled peptide is

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converted to the amyloid form. Thus, it appears that the amyloid fibril is constructed by the accumulation of self-recognition, self-capturing and conformational self-replication, based on its self-complementary sequence. Although detailed mechanisms by which the abnormal isoform of prion protein (PrP^{Sc}, amyloid form) propagates are not fully understood, it seems to be consenting that PrP^{Sc} catalyzes a conversion of the protein conformation from its normal isoform (PrP^{C}) to PrP^{Sc} without any chemical modification. That is, PrP^{Sc} conformationally self-replicates, resulting in the self-amplification of PrP^{Sc}.[3] It should be noted that the term 'self-replication' or 'autocatalysis' often used in cases of amyloid proteins means that they self-replicate or autocatalyze conformationally but not chemically.

In this study, we have attempted to design an artificial system to explore applicability of using the amyloid-like fibril as a construct possessing molecular recognition and catalytic abilities. Here, we especially focused on the self-recognition ability of fibrils, because the self-complementarity required for the recognition can be easily generated by manipulating the amino acid sequences.[1d–f] The self-recognition of various kinds of molecules has been widely applied to construct artificial molecular systems,[15] in which the chemical self-replication of oligonucleotides,[16] peptides,[17,18] micelles[19] and abiological organic molecules[20] has been demonstrated. Molecular replication is seen in systems where the product serves as a catalyst for its own synthesis. Therefore, this function has been discussed in relevancies to molecular evolution of prebiotic polymers. Thus, the self-replicating process is based on the recognition of self-complementary structures. So far, artificial self-replicating systems of peptides have been reported, in which α-helical coiled-coil motifs (associated amphiphilic α-helices) have been employed.[17,18] These studies showed that the peptide was able to catalyze its own synthesis by accelerating the amide bond condensation between fragments by the recognition process. In this system, hydrophobic amino acids define a complementary interhelical recognition surface, initiating the association of the peptide fragments and accelerating the condensation reaction by the native chemical ligation.[21]

This paper describes demonstration to construct a self-replicating system of a peptide to form amyloid-like fibrils by employing the native chemical ligation strategy, in which the peptide self-replicates not only conformationally but also chemically (Fig. 1). This novel reaction was based on the self-complementary recognition ability of the fibrous β-structure.[1] The self-recognition and self-replication reactions of simple polypeptides will inspire the idea of molecular evolution in the prebiotic era.

2. Results and discussion

2.1. Design and synthesis

It is considered that the preformed amyloid fibril has a role of a template acting to organize other participating

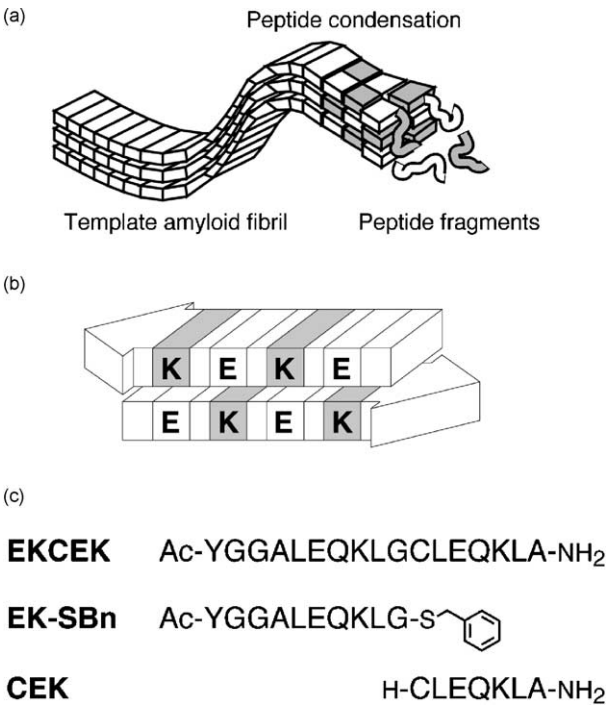

Figure 1. Structures and schematic representation of self-replicating peptides employing the chemical ligation reaction; (a) Illustration of chemical and conformational self-replication of amyloid fibrils; (b) self-complementary recognition of the peptide in an antiparallel β-sheet;[1e,f] (c) The primary structures of the full length peptide (EKCEK), the N-terminal fragment (EK-SBn) and the C-terminal fragment (CEK).

free peptide subunits, which may be present in a different conformation from the template fibril such as α-helix or unfolded structure (Fig. 1). If a similar template effect can also operate on shorter complementary peptide fragments, it may constitute the basis of an autocatalytic cycle for peptide self-replication. It can be a demonstration of a chemical and conformational self-replication of amyloid fibrils, if the following system is achieved. Preorganization of the reactants (fragment peptides) on the template amyloid occurs at first based on the self-complementarity recognition, which promotes the condensation of the fragment peptides, and then the template amyloid organizes the newly-generated peptide (chemically identical copy of the template) on the template in association with the promotion of (re)folding to the β-sheet. These steps result in the self-amplification of the fibrils. In contrast to the self-replicating systems employing coiled-coil peptides[17,18] and also ordinary enzymatic catalyses, it is expected that the present system employing amyloid-like fibril would not require the dissociation of reacted substrates to maintain the template effect and catalytic activity.

A peptide that can assemble into amyloid-like fibrils and thus act as a template was designed on the basis of the peptides presented in the reported paper (Fig. 1).[1] It has been shown that the sequence of ALEQKLAA-LEQKLA (parent EKEK sequences) has a potential to form the β-sheet fibril, that is, it has the self-complementarity required for the self-assembly into amyloid-like fibrils.[1] Based on this parent sequence, a

peptide sequence ALEQKLG-CLEQKLA (EKCEK) was designed as the putative template amyloid (Fig. 1). The amino acids at positions 10 and 11 were chosen as the site for breaking the full sequence to form the N-terminal and C-terminal fragments and Gly and Cys residues were, respectively, used at the positions. This ligation site was chosen because it is the middle position of the core sequence, and effects of amino acid substitutions in these positions on the amyloid formation of the peptide were expected to be relatively low.[1] According to the peptide fragment condensation strategy,[21] the N-terminal peptide fragment was preactivated as a thiobenzyl ester and the C-terminal fragment was equipped with a free Cys residue at the N-terminus. Such a coupling strategy circumvents the need for addition of external coupling reagents to the reaction mixture. The Ala-to-Gly substitution at position 10 was made in order to increase the flexibility of thioester site. The three residues YGG were introduced at the N-terminus to provide a spectroscopic handle, as well as to facilitate the peptide aggregation by its hydrophobic character.[1c] Consequently, the full length peptide (EKCEK), its N-terminal (EK-SBn) and C-terminal fragments (CEK) were designed (Fig. 1).

All peptides were synthesized by the solid-phase method by means of 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry.[22] The C-terminal peptide was prepared on Rink amide resin,[23] and the N-terminal thioester fragments were synthesized on a 2-chlorotrityl chloride resin,[24,25] which provided the C-terminal free peptide having all side-chain protecting groups. The protected peptide was coupled with benzyl mercaptan (BnSH), and then the protected thioester peptide was treated with trifluoroacetic acid (TFA) to give the peptide thioester. All peptides used were purified by reversed-phase HPLC (RP-HPLC) to give a high purity, and identified by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) and amino acid analysis. The amino acid analyses were also utilized for determination of the peptide concentration of the stock solutions.

2.2. Amyloid-like fibril formation of the template peptide

Conformational analyses of the full-length peptides EKCEK were carried out by circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopies, and amyloid-like fibril formation was characterized by an amyloid-specific dye thioflavin T (ThT) binding analysis and transmission electron microscopy (TEM). The analysis of the time-dependent fibril formation was carried out by using the fluorescent dye, ThT, which associates with fibrils, and the binding results in a significant increase in the fluorescence depending on the amount of fibrils present.[1a,11] CD studies revealed that EKCEK (50 μM) was in almost random coil shortly after dissolution in the neutral buffer. To induce the fibril formation of EKCEK, it was incubated at a peptide concentration (0.9 mM) and a temperature (40 °C), both conditions are higher as compared with those reported in the previous paper.[1a] Using these conditions for 3 days, ThT with the incubated EKCEK showed the fluorescence spectra with a new excitation maximum at ~440 nm and an enhancement in emission at ~480 nm (Fig. 2a), which was a characteristic spectrum for ThT bound to amyloid fibrils.[1,11] The fibril formation of the peptide was confirmed by TEM observation (Fig. 2b). In addition, CD and FTIR measurements revealed the formation of a β-sheet structure after the incubation (single negative maximum at 218 nm in the CD spectrum (Fig. 2c) and amide I absorption maximum at 1623 cm^{-1} in the FTIR spectrum).[1a,26] The Cys residue of the peptide in the fibril form, however, was almost completely oxidized to form the intermolecular disulfide bond, resulting in the dimeric peptide, after the transition. The ThT-binding and HPLC analyses revealed that after the peptide formed the fibrils in the absence of reductant, the addition of 1% BnSH (v/v) almost completely reduced the disulfide bond to the free thiol within 1 h without disruption of the preformed fibrils. This BnSH-reduced fibrils formed by EKCEK were used as the template in the fragment condensation reaction.

2.3. Ligation reaction in the absence of the template fibrils

The thioester-promoted amide bond formation between the fragment peptides to produce the full length EKCEK peptide was examined. The ligation reaction of N-terminal fragment (EK-SBn, 190 μM) and the C-terminal fragment (CEK, 190 μM) in 250 mM MOPS buffer (pH 7.5) containing 1% BnSH (v/v) was monitored by an RP-HPLC analysis as shown in Figure 3. The ligation products were identified by MALDI-TOFMS

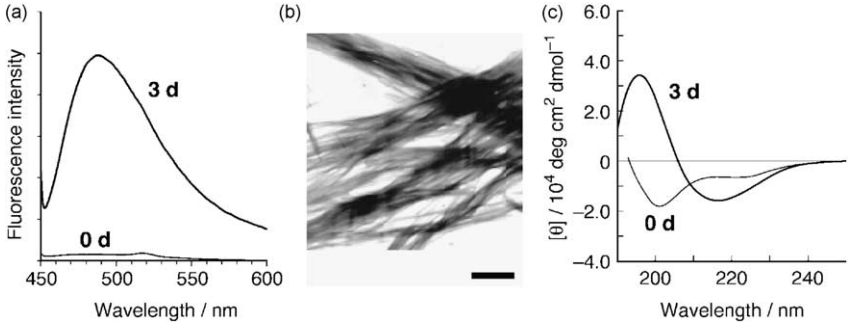

Figure 2. Conformational transition and fibril formation of the peptide EKCEK: (a) ThT-binding analysis of EKCEK. Fluorescence spectra of ThT (λ_{ex} = 430 nm) 0 and 3 days incubation at 40 °C. (b) Transmission electron micrograph of the fibrils formed by EKCEK. Magnification, 50,000×; scale bar, 200 nm. (c) Conformational transition elucidated by CD spectra at 25 °C after 0 and 3 days incubation at 40 °C.

and with authentic samples on HPLC. The ligation reaction was found to be highly regio- and chemoselective. When this ligation reaction of EK-SBn and CEK was performed in the presence of 6 M guanidine hydrochloride (GuHCl), the reaction rate was remarkably decreased (Fig. 3c). The reaction rate in the presence of the denaturant can be assumed to reflect a background reactivity of the thioester-promoted amide bond formation, in which contribution of the peptide secondary structure to the reactivity can be excluded. Thus, the condensation reaction of EK-SBn and CEK in the non-denaturing conditions was intrinsically enhanced compared with the background level. This indicates the structural character of EKCEK having a potential to self-assemble intermolecularly.

2.4. Ligation reaction in the presence of the template fibrils

The condensation reaction of EK-SBn and CEK were carried out in the presence of full-length peptide EKCEK to examine a template effect of preformed fibrils. The template peptide was preincubated to give the amyloid-like fibrils. In the presence of the template EKCEK fibrils (initial concentration, 80 μM), the ligation reaction was started with initial peptide concentrations of 190 μM EK-SBn and 190 μM CEK. The reaction was enhanced apparently as compared with that in the absence of template fibrils (Fig. 3d). Although an effective autocatalysis should be characterized by the rate enhancement as the initial template concentration increases,[17] further increased initial concentration of the template fibrils (up to 200 μM) did not lead to an apparent rate enhancement. The template effect of EKCEK in the fibril form might be limited in the macromolecular assemblage and thus effective molar concentration of the template catalyst (substrate-accessible surface) is extremely decreased. It is also considered that the concentration of the catalytic site is almost constant independent to the peptide concentration. However, when the ligation reaction was carried out in the presence of EKCEK (80 μM), which was not preincubated (i.e., non-fibril form), no effect on the reaction rate was observed, the product formation was comparable to that without the template (Fig. 3d). This result suggested that the conformationally disordered monomeric species was not effective, but that the preformed fibril structure is important to act as the template. That is, the template effect (substrate recognition and its organization) is due to the well-organized tertiary structure of the amyloid-like fibrils.

2.5. Conformational self-replication of the amyloid-like fibril

The increase of amyloid-like fibrils in the ligation reaction system was examined by the ThT-binding analysis (Fig. 4). The fluorescence intensity of ThT in the presence of the mixture of EKCEK fibrils and the fragments, EK-SBn and CEK, was increased in time-dependent compared with that in the case of EKCEK alone (without the substrate fragments). It was revealed that the amount of the fibrils increased as the fragment condensation proceeded to generate EKCEK. In contrast, when the condensation reaction was carried out in the presence of the non-preincubated EKCEK (non-fibril form), no fluorescence increase was observed at this low concentration of the peptide. It could be concluded that the amyloid-like fibrils were conformationally self-amplified by converting the conformation of the newly generated peptide to that identical to the preexisting fibrils. These results imply that both chemical and

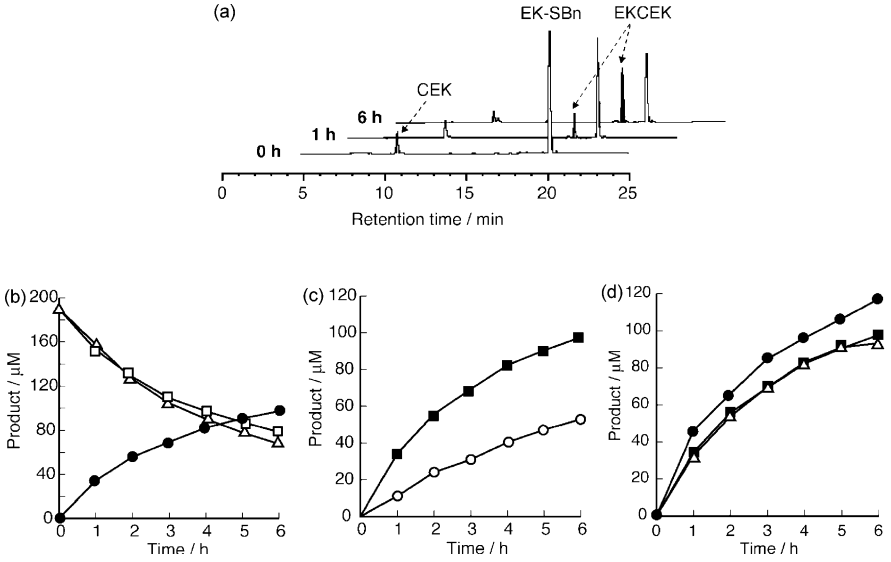

Figure 3. Ligation reaction of EK-SBn and CEK: (a) RP-HPLC profiles of the reaction mixture at the indicated time after the initiation of the reaction, monitored at a wavelength of 220 nm; (b) time-dependent production of the full length peptide (EKCEK, closed circle) with plots for the N-terminal fragment (EK-SBn, triangle) and the C-terminal fragment (CEK, square); (c) production of EKCEK in the presence (open circle) or the absence (closed square) of 6 M GuHCl; (d) effects of the preexisting EKCEK peptide on the condensation reaction. The reaction of the N-terminal fragment (EK-SBn) and the C-terminal fragment (CEK) was carried out in the presence of the pre-incubated (fibril form) EKCEK (closed circle) or non-pre-incubated (random coil) EKCEK (closed square), and the absence of the preexisting EKCEK (triangle). $[EK\text{-}SBn]_0 = 190$ μM and $[CEK]_0 = 190$ μM with or without $[EKCEK]_0 = 80$ μM in 250 mM MOPS buffer (pH 7.5)/1% BnSH (v/v) at 30 °C.

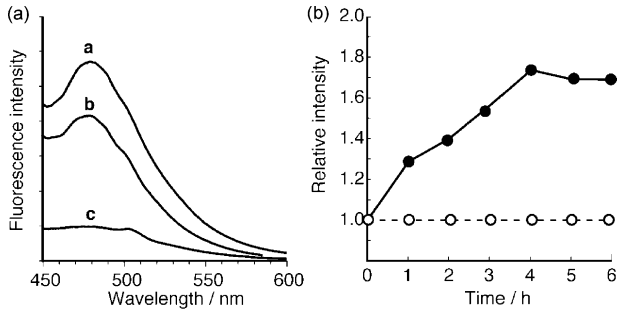

Figure 4. Conformational self-replication elucidated by the ThT-binding analysis of the ligation reaction: (a) fluorescence spectra of ThT ($\lambda_{ex} = 430$ nm) in the presence of the reaction mixtures with the pre-incubated EKCEK (fibril form) at 3 h (**a**) and 0 h (**b**), and with non-pre-incubated EKCEK (random coil) at 3 h (**c**); (b) time courses of the relative ThT-fluorescence intensities at 480 nm of the self-replication reaction with the pre-incubated EKCEK (fibril form) (closed circle). The values were represented by the ratio of those of reaction mixture to those of the template EKCEK (fibril form) alone without any fragment peptides (open circle) at each reaction time. The reaction conditions are same as described in Figure 3.

conformational self-replication of amyloid-like fibrils was accomplished.

3. Conclusion

This study showed a novel attempt to construct a self-replicating system of peptides to form amyloid-like fibrils, in which peptide self-replicates not only conformationally but also chemically, although the total catalytic efficiency was not high. This reaction was based on the self-complementarity recognition between peptides. The template fibrils organize the newly generated peptide (chemically identical copy) on the template, in conjunction with the promotion of folding to the fibril form (conformationally identical copy). Thus, it leads to the self-amplification of the fibrils.

In designing the present system, we especially focused on the self-recognition ability of the amyloid-like fibrils,[1e,f] and employed the ligation reaction to generate a native peptide bond. The present system is a demonstration of applicability to utilize the amyloid-like fibril as a macromolecular construct possessing molecular recognition and catalytic abilities. It will be a promising strategy to employ the intrinsic molecular recognition abilities of the amyloid-like fibril in addition to the highly organized tertiary structure and chiral surface. On the basis of such features of this macromolecular assemblage, construction of novel functional/catalytic amyloid fibrils may be possible by the molecular design of amyloidogenic peptides, which will be further expanded by the amyloid architecture methodology.[1e,f]

On the other hand, peptide amyloid is thought to be one of the common characters of polypeptide, because peptides and proteins that have different primary and tertiary structures form such a common fibrous structure.[2] If the peptide amyloid could contain a nature of polypeptide in a prebiotic era, this self-catalytic function demonstrated in this study may show a relevance of molecular evolution of prebiotic macromolecules. This kind of consideration is interesting to generate a novel functional molecule on the basis of molecular evolution, which is different from that with RNA.

4. Experimental

4.1. Peptide synthesis

Peptides were synthesized manually or automatically by the solid-phase method of Fmoc chemistry.[22] The full length peptides (EKCEK) and the C-terminal fragment peptides (CEK) were synthesized on Rink amide resin[23] using Fmoc-amino acid derivatives with the side chain protections; *t*-butyl for Glu and Tyr, Boc for Lys, and triphenylmethyl (Trt) for Gln and Cys. The peptides were purified by semi-preparative RP-HPLC to give a single peak on an analytical RP-HPLC, and characterized by MALDI-TOFMS and amino acid analysis. MALDI-TOFMS: found $[M+H]^+$ (calcd $[M+H]^+$); EKCEK, 1863.7 (1863.2); CEK, 803.5 (804.0).

The N-terminal thioester peptides were assembled on a 2-chlorotrityl chloride (Cl-Trt) resin[25] using Fmoc-amino acid derivatives as described above. The cleavage of peptide from resin without removal of any side chain protecting group was carried out with acetic acid (AcOH)/trifluoroethanol (TFE)/DCM (1/1/3).[25b] For the thioesterification, the protected peptide was treated with benzyl mercaptan (BnSH, 3 equiv), 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDCI·HCl, 3 equiv) and HOBt·H_2O (3 equiv) in DMF for 1 day to give the fully-protected benzyl thioester peptide. All the side chain protecting groups were removed with 95% TFA/H_2O. The final compounds (EK-SBn) was purified by RP-HPLC, and identified by MALDI-TOFMS and amino acid analysis. MALDI-TOFMS; found $[M+H]^+$ (calcd $[M+H]^+$); 1184.4 (1184.4).

4.2. Pre-incubation of the template peptides for the fibril formation

The template peptide (EKCEK) (0.87 mM) in 250 mM MOPS buffer (pH 7.5) was incubated at 40 °C for 3 days, after which the amyloid-like fibril formation was examined by ThT-binding[1,11] and TEM analyses. For the ThT-binding analysis, to 20 μL of the incubated solution were added 10 μL of the ThT stock solution (240 μM in water) and 370 μL of 250 mM MOPS buffer (pH 7.5) (finally 44 μM peptide and 6 μM ThT), and immediately the excitation ($\lambda_{em} = 480$ nm) and emission spectra ($\lambda_{ex} = 430$ nm) were recorded. Fluorescence spectra were measured on a Hitachi F-2500 fluorescence spectrophotometer at 25 °C using a 5×5 mm quartz cell. The fibril formation was also characterized with a Hitachi H-7500 electron microscope operating at 100 kV (negative staining, a 2% (w/v) aqueous phosphotungstic acid solution). Prior to using the peptide as the template in the ligation reaction, this preincubated peptide solution was diluted with the buffer containing 1% (v/v) BnSH, and the solution was hold for 1 h. By this treatment, the disulfide bond was almost completely reduced without disturbing the fibril form, which was

examined by RP-HPLC and ThT-binding analyses. The peptide samples preincubated by this method were used as a template for the ligation reaction with dilution of fibrils to 80 μM. Non-preincubated samples at 80 μM cannot make fibrils under the conditions used here.

4.3. Circular dichroism spectroscopy

For the CD measurements of the incubated EKCEK solution (0.87 mM peptide at 40 °C for 3 days), it was further 50-fold diluted with water to give the 17 μM peptide concentration at the measurements. CD spectra were recorded on a J-720WI spectropolarimeter at 25 °C using a quartz cell with 1.0 mm pathlength.

4.4. FTIR spectroscopy

The peptide solution was prepared by diluting the stock solution with the buffer and incubated as described above (0.87 mM peptide at 40 °C for 3 days). The peptide solution (50 μL) was dropped on a CaF_2 plate and the solution was air-dried, then in vacuo. Spectra were recorded on a Perkin–Elmer 1600 spectrophotometer at 4 cm^{-1} resolution.

4.5. Ligation reaction

All reactions were started by adding the stock solutions [9.5 mM in H_2O/acetonitrile (1/1)] of the N- and C-terminal fragment peptides to 250 mM MOPS buffer (pH 7.5) containing 1% BnSH (v/v). Temperature was maintained at 30 °C. At the indicated time interval, aliquots (50 μL) were taken from the reaction mixture, immediately quenched with 5% aqueous TFA containing 8 M GuHCl (50 μL), and analyzed by RP-HPLC on a Cosmosil 5C18-AR-II (4.6×150 mm) column using a linear gradient of acetonitrile/0.1% TFA (10–60% over 25 min) at a flow rate of 1.0 mL min^{-1}. The samples were stored at −20 °C prior to HPLC analysis. The reaction products were identified by MALDI-TOFMS and with the authentic samples on HPLC.

4.6. Thioflavin T binding analysis of ligation reaction

The ligation solution containing the template peptide was prepared ($[EKCEK]_0 = 80$ μM, $[EK\text{-}SBn]_0 = 190$ μM and $[CEK]_0 = 190$ μM in 250 mM MOPS buffer (pH 7.5) containing 1% BnSH). At the indicated time interval, aliquots (50 μL) were taken from the reaction mixture, 10 μL of the ThT stock solution (240 μM in water) and 340 μL of 250 mM MOPS buffer (pH 7.5) were added (finally 21 μM EKCEK, 24 μM EK-SBn, 24 μM CEK and 6 μM ThT), and the excitation ($\lambda_{em} = 480$ nm) and emission spectra ($\lambda_{ex} = 430$ nm) were recorded immediately at 25 °C using a 5×5 mm quartz cell.

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