

# Novel Methods for Inhibiting Amyloidogenesis in the Presence of Peptides to Block Hydrophobic Interactions

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## Abstract

Amyloid fibrils, which are caused by abnormal conformation and the mis-assembly of proteins, are responsible for several conformational diseases, including prion diseases. To develop methods to prevent amyloid formation, blocking peptides with hydrophilic substitutions covering the stem forming regions of barnase 1-24 were prepared and examined for their ability to block amyloid-forming fragments—prion, Amyloid  $\beta$ , Pmel 17—. When these fragments were mixed with the synthetic blocking peptides, the result was a decline in the intensity of fluorescence, suggesting that amyloid formation was inhibited. Therefore, amyloidogenesis appears to be specifically inhibited by disrupting the hydrophobic interactions between core amyloid regions.

## Introduction

The formation of amyloid fibrils is responsible for several disorders including Alzheimer's disease, prion disease, and dialysis amyloidosis, which are collectively known as "conformational diseases" [1]. Amyloid fibrils play a role in many neurodegenerative diseases [2]. On the other hand, functional polymers similar to amyloid-like fibrils are formed by the transmembrane protein Pmel17 in melanosomes [3–13]. Although there is wide variation in the native three-dimensional structures, protein-forming amyloids are typically 10 nm in diameter, long, and unbranched [14–19], and can be identified by their unique ability to bind to the dyes thioflavin T (ThT) and Congo red. The fibril structure has recently been clarified in detail by cryo-electron microscopy [20].

Insight into mechanisms that inhibit amyloidogenesis is crucial for the therapeutic treatment and clinical analysis of amyloid-related disease [21]. Recently, Griner et al. [22] designed peptide-based inhibitors that reduce both amyloid  $\beta$  (A $\beta$ ) aggregation and the toxicity of aggregated species. Despite the common morphology of amyloid fibrils, however, there is no general inhibitor of amyloidogenesis.

To develop methods for preventing amyloid formation, it is necessary to inhibit the aggregation of  $\beta$ -structures [23, 24]. Recently, it was shown the approach to bind such segments in  $\beta$ -strand and  $\beta$ -hairpin conformations using de novo

designed scaffolds [25]. Previously, we examined intermolecular interactions among several amino acid residues in barnase1-24 (BM1-24), a protein known to form amyloid-like fibrils [26]. Using a series of mutated barnase molecules, we identified interactions between hydrophobic residues on both sides of the  $\beta$ -strand (Fig. 1a-c) that are essential in fibril formation. In the present study, to block the hydrophobic interactions between hydrophobic residues on both sides of a  $\beta$ -strand, two peptides (SS-1, SS-2) were prepared in which residues on only one side of the  $\beta$ -strand, located at even numbers, were substituted by hydrophilic residues (Fig. 1d-f). A negative control (NC) peptide, possessing hydrophobic residues on both sides of the  $\beta$ -strand, was also prepared.

## Materials and methods

### Peptide synthesis

Peptides were prepared by solid-phase peptide synthesis using Fmoc strategy as previously described

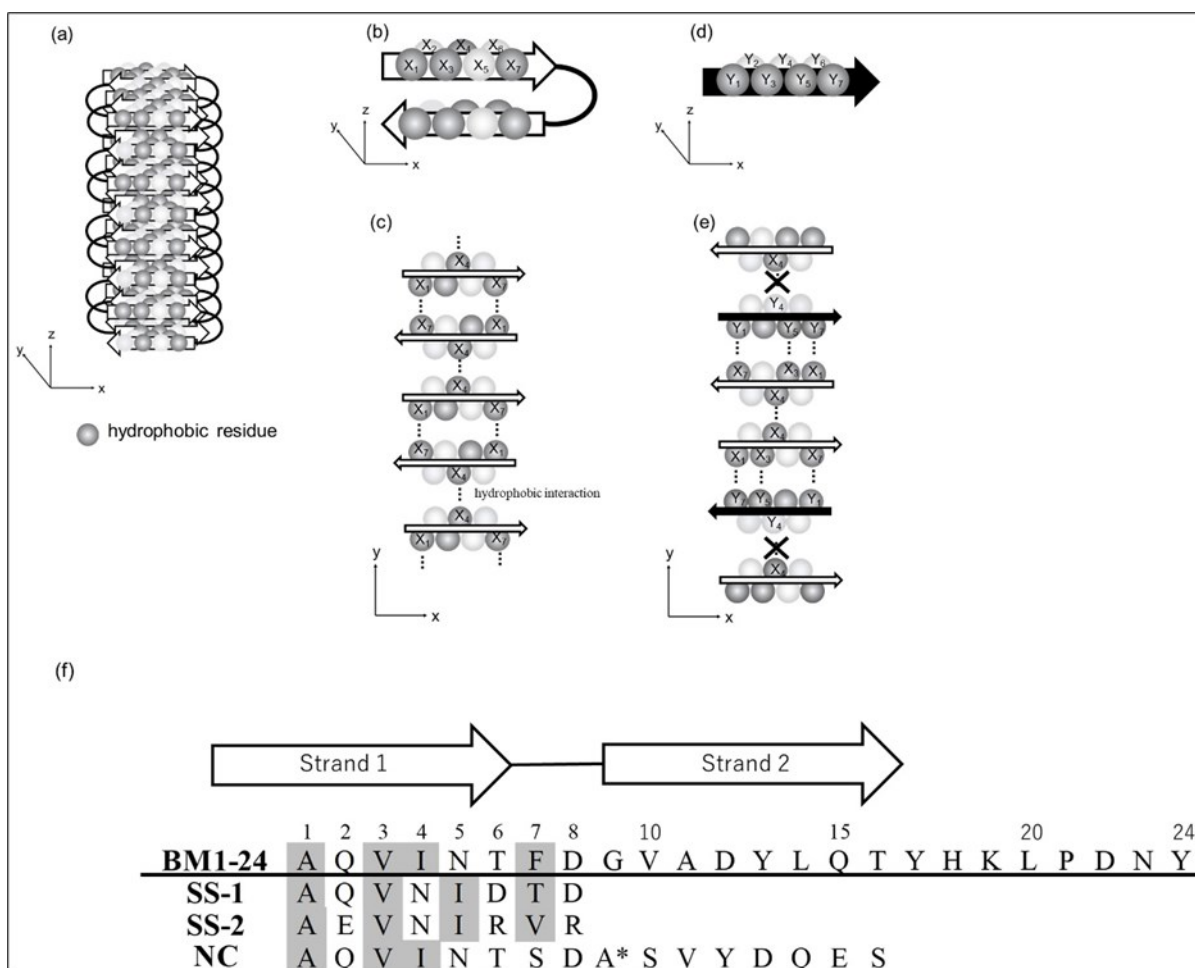


Figure 1. Model summarizing the strategy for preventing amyloid formation by blocking interactions in amyloid core regions. Dark gray balls represent hydrophobic residues. (a) Oblique projection of part of an amyloid fibril. (b) Antiparallel  $\beta$ -sheet with an even-length of  $\beta$ -strands for amyloid formation. (c) Cross-sectional view of an amyloid fibril. Dotted lines represent hydrophobic interactions between neighboring protofibrils. (d) Designed  $\beta$ -strand for blocking amyloid formation. (e) Cross-sectional view showing the blocking amyloid formation. (f) Amino acid sequences of the synthetic peptides. The sequence of BM1-24 is given in the first row. The special sequence, DA\*, where A\* denotes D-Ala, is designed to induce a turn. Hydrophobic residues are highlighted in gray.

[27]. In brief, peptides with a C-terminal amide group were assembled on Fmoc-NHCH<sub>2</sub> Ph(OCH<sub>3</sub>)<sub>2</sub>-O-resin obtained from Rapp Polymere. The subsequent coupling of Fmoc-protected amino acids was carried out using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and N-hydroxybenzotriazole (HOBt). In each synthetic cycle, the terminal Fmoc group was removed by DMF solution containing 1.1% 1,8-diazabicyclo[5.4.0]-7-undecene, 7.7% piperidine, and 2.3% 1-hydroxybenzotriazole. The protecting group and resin were removed by shaking the peptidyl resin in trifluoroacetic acid containing 5% triisopropylsilane and 3% water for 1.5 h at room temperature. The reaction mixture was poured into cold diethyl ether, and the precipitated peptide was collected. The product was taken up by dissolving the mixture in 20% aqueous solution of acetic acid and filtering the mixture to remove the resin beads. RP-HPLC was used to purify the peptides.

#### *Fibril formation*

Each lyophilized synthetic peptide (SS-1, SS-2, and NC) was solubilized at 0.2 mM in 50 mM Tris chloride buffer (pH 7.5) in a microtube. Lyophilized peptides of BM1-24, prion180-193, Amyloid  $\beta$ , and serum amyloid A protein 1-27 (SAA1-27) were prepared in the same solution, whereas Pmel17 405-420 was solubilized in sodium acetate buffer (pH 6.0). These solutions were admixed with SS-1, SS-2, or NC, incubated for 7 d under static conditions at 4°C, and then analyzed by ThT assay and CD spectroscopy.

#### *Thioflavin T assay*

The thioflavin T (ThT) assay was used for the detection of fibril formation by measuring ThT fluorescence enhancement that occurs in the presence of fibrils. Synthetic peptides were prepared by adding 20 mL of incubated peptide solution to 2 mL of aqueous ThT. The final concentrations of the peptide and ThT were 2 mM and 5 mM, respectively. The formation of amyloid fibrils was monitored by fluorescence enhancement of fibril-bound ThT in 50 mM Tris buffer (pH 7.5). Fluorescence emission spectra were collected in the range 460–600 nm with an excitation wavelength of 450 nm as previously described [13]. Fluorescence enhancement of ThT in the amyloid-bound state,  $\Delta F$ , was defined as  $\Delta F = (F_S - F_0) / F_0$ , where  $F_S$  and  $F_0$  denote the fluorescence intensity of the sample and that of the control solution without peptides, respectively.

#### *Circular dichroism spectroscopy*

A circular dichroism (CD) spectrum was recorded in the far-UV region (200–260 nm) at 20°C with a JASCO J-725 spectropolarimeter, a quartz cuvette, and a 1.0 mm path length. The spectral data were recorded in terms of mean residue ellipticity,  $[\theta]$ , in degrees square centimeter per decimole.

## **Results and Discussion**

### *Peptide Inhibition of BM1-24 Amyloidogenesis*

The ability of BM1-24 to form fibrils in the presence or absence of the synthetic blocking peptides (SS-1 and SS-2) and the negative control peptide (NC) was evaluated by ThT assay, as shown in Fig. 2. Based on a previous report [26–28], a change in fluorescence intensity ( $\Delta F$ ) of  $>1$  was considered to indicate a significant amount of amyloid formation. The solution of BM1-24 alone underwent considerable amyloid formation, as shown by the  $\Delta F$  value of  $>3$ . In contrast, adding SS-1 or SS-2 to the BM1-24 solution resulted in much a lower fluorescence intensity ( $\Delta F < 0.5$ ), suggesting that amyloid formation was inhibited. Furthermore, the NC peptide comprising hydrophobic residues on both sides of the  $\beta$ -strand was unable to inhibit amyloid formation, suggesting that one side of the BM1-24  $\beta$ -strand is formed by hydrophilic residues.

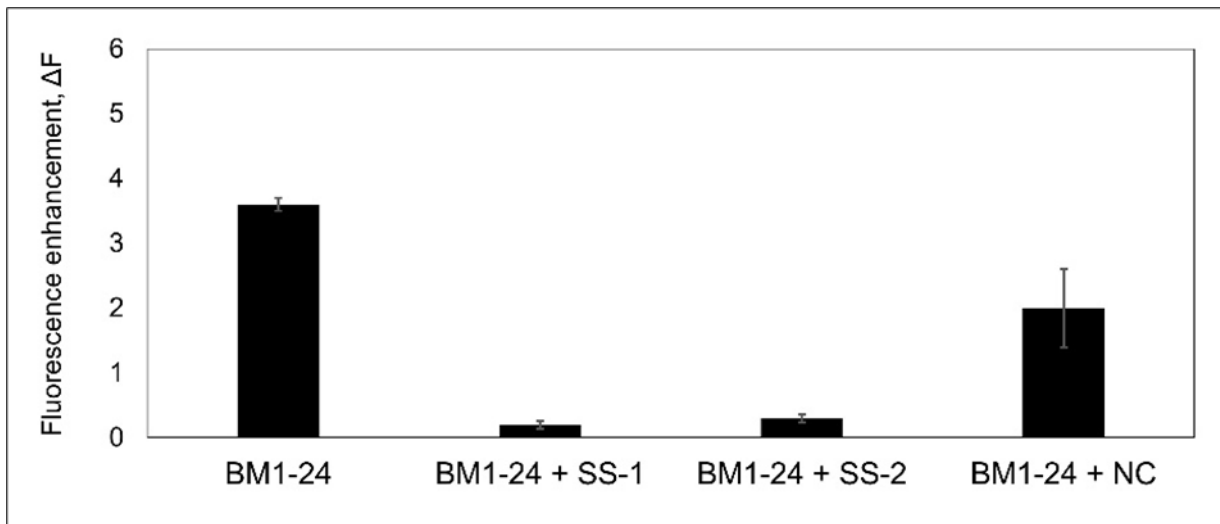


Figure 2. Thioflavin-T assays showing amyloid formation of BM1-24 and the mixture of BM1-24 with synthetic blocking peptides.

To obtain structural information related to fibril formation of BM1-24 in the presence and absence of SS-1, we carried out CD measurements under the same biochemical conditions as the ThT assay (Fig. 3). No significant secondary structure was observed in the SS-1 peptide. By contrast, BM1-24 exhibited a characteristic CD pattern with a negative peak at 207 nm, as reported previously [26,29]. In addition, BM1-24 in the presence of SS-1 produced a CD spectrum with a negative peak at 218 nm,

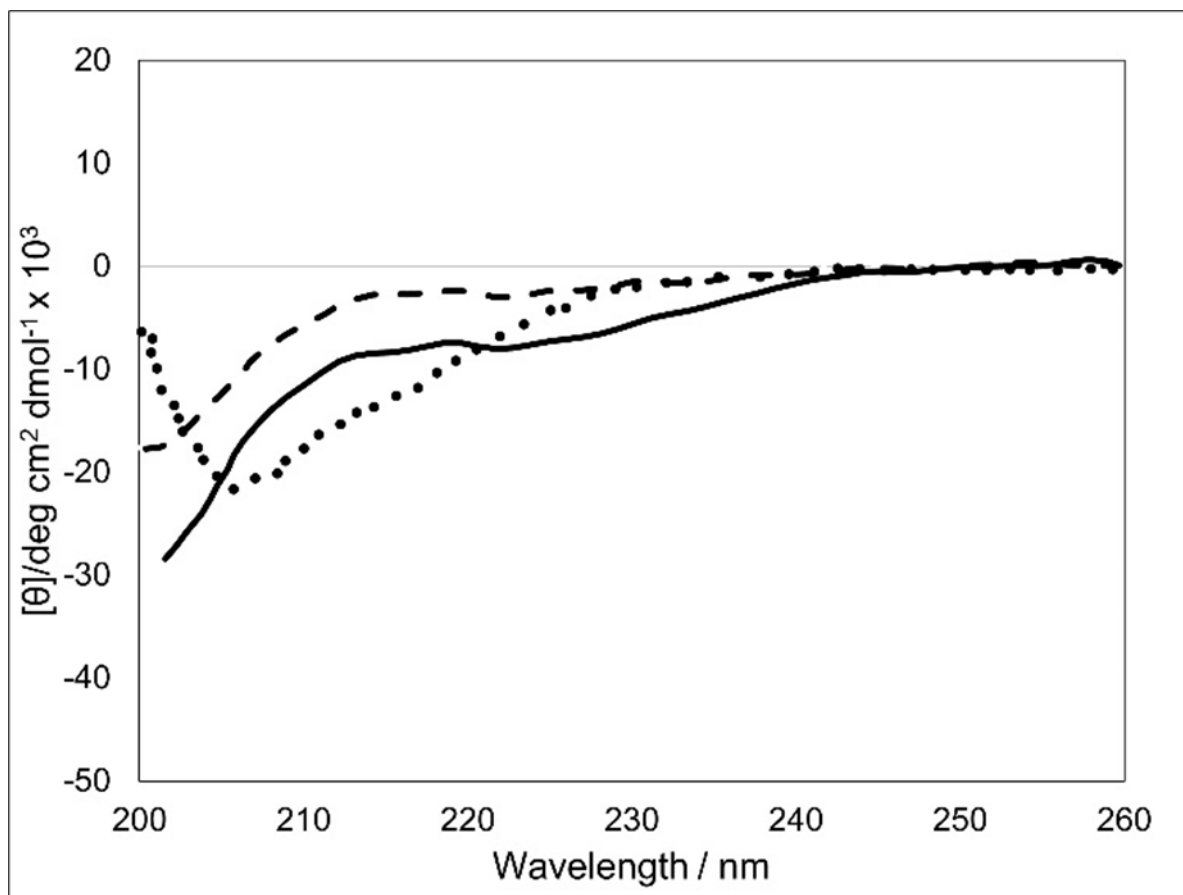


Figure 3. CD spectra of SS-1, BM1-24, and BM1-24 in the presence of SS-1. Dashed line, SS-1; dotted line, BM1-24; solid line, BM1-24 plus SS-1.

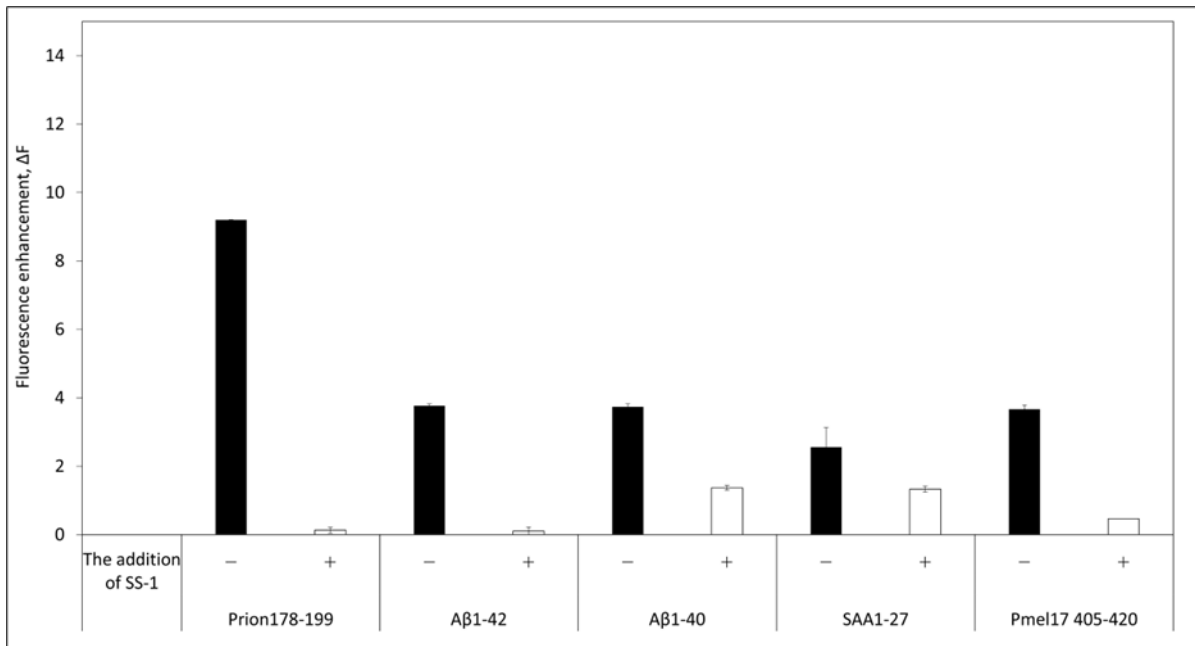


Figure 4. Thioflavin-T assays of amyloid fragments in the absence (open bars) and presence (shaded bars) of SS-1.

which is consistent with a  $\beta$ -sheet-like structure. It is possible, therefore, that addition of the SS-1 peptide to BM1-24 may have stabilized the  $\beta$ -structure of BM1-24 with hydrophobic residues of SS-1 being specifically recognized by hydrophobic residues of BM1-24.

#### *Peptide Inhibition of General Amyloid genesis*

Next, we investigated whether the SS-1 peptide might be applicable to the inhibition of other proteins that are known to form amyloid fibrils. We prepared peptides of the following amyloid-forming proteins, prion180-193 [27], Amyloid  $\beta$ , serum amyloid A (SAA) protein 1-27, and Pmel17 405-420 [30] by chemical synthesis and assessed amyloidogenesis in the presence of SS-1 by ThT fluorescence assay. As shown in Fig. 4, addition of SS-1 to each of the amyloid-forming fragments led to a decline in fluorescence intensity, suggesting that amyloid formation had been inhibited. Therefore, our results indicate that the SS-1 peptide is effective at inhibiting amyloidogenesis in various types of protein through the formation of hydrophobic interactions.

### **Conclusions**

Herein, we have presented a structural model to design peptides for blocking amyloidogenesis via the formation of hydrophobic interactions. The SS-1 peptide, comprising eight amino acids, was designed to have hydrophilic residues (Gln, Glu, Asn, and Asp) on only one side of the  $\beta$ -sheet. In addition to inhibiting amyloid formation by BM1-24, SS-1 blocked amyloidogenesis by peptides of prion protein, amyloid- $\beta$ , SSA, and Pmel17. In summary, amyloidogenesis seems to have been specifically inhibited by the disruption of hydrophobic interactions between core amyloid regions. Our newly designed peptide may be useful for analyzing amyloid  $\beta$  aggregate formation and for studying diseases associated with the formation of amyloid fibrils (amyloidosis). Further optimizing the design of inhibitory peptides and verifying their effectiveness in *in vivo* experiments will likely lead to the development of more effective amyloid fibril inhibitors.

### Abbreviations

CD - Circular dichroism; NC - Negative control

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