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Inhibition of Aβ42 aggregation using peptides selected from combinatorial libraries

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Increasing evidence suggests that the aggregation of the small peptide Aβ42 plays an important role in the development of Alzheimer's disease. Inhibiting the initial aggregation of Aβ42 may be an effective treatment for preventing, or slowing, the onset of the disease. Using an *in vivo* screen based on the enzyme EGFP, we have searched through two combinatorially diverse peptide libraries to identify peptides capable of inhibiting Aβ42 aggregation. From this initial screen, three candidate peptides were selected and characterized. ThT studies indicated that the selected peptides were capable of inhibiting amyloid aggregation. Additional ThT studies showed that one of the selected peptides was capable of disaggregating preformed Aβ42 fibers. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article

Keywords: peptide libraries; Aβ42; Thioflavin T; Alzheimer's disease; amyloid inhibition

Introduction

Many proteins are known to adopt alternative misfolded structures that can be linked to a variety of diseases [1–3]. These alternative structures no longer retain the function associated with the native structure of the protein, and thereby become functionally useless or toxic to the cell. Misfolded proteins are capable of aggregating together to yield a variety of oligomeric states, ultimately forming fibers and amyloid plaque. The formation of fibers and amyloid in human tissue appears to be a nearly irreversible reaction.

While resolubilizing amyloid appears to be very difficult to achieve, there has been some success made in slowing the rate of aggregation, if not preventing aggregation altogether. Several chaperon-like proteins, such as clusterin and HSP20, as well as several antibodies have been shown to be capable of preventing amyloid formation [4–10]. Likewise, several small molecules and peptides have been shown to slow the formation of amyloid [11–26]. Recently, Sato *et al*. synthesized several peptides found to inhibit the aggregation of Aβ42 [27]. These peptides synthesized by Sato and coworkers were rationally designed to bind to, and inhibit the aggregation of, Aβ42.

The use of small peptides as aggregation-inhibiting agents could prove therapeutically useful for a variety of diseases, including Alzheimer's disease. Slowing the progress of drug discovery for Alzheimer's disease is the lack of an inexpensive and easily accessible screen. Most of the screens that have been reported make use of synthetic Aβ42 [28,29]. The synthesis of Aβ42 is time-intensive and prohibitively expensive for use in screens of peptide libraries. Additionally, synthetic Aβ42 has an extremely high propensity to form homo-oligomeric complexes in solution. These initial oligomers act as seeds to promote the formation of amyloid. Because of the difficulty in removing the seed oligomers, screens aimed at finding compounds that prevent the earliest stages of amyloid formation are likely to fail.

Here, we describe an EGFP-based screen, developed by Hecht and coworkers [30–32], to select for small peptides capable of inhibiting the aggregation of Aβ42. Two peptide libraries were constructed to associate with, and prevent the aggregation of, Aβ42. In this screen, the peptide Aβ42 was genetically fused to EGFP. When expressed in *Escherichia coli*, the Aβ42-EGFP fusion protein produces virtually no green color or fluorescence due to the amyloidogenic nature of Aβ42. Aggregation of Aβ42 precludes folding, and hence fluorescence, of the EGFP reporter. However, when Aβ42 is prevented from aggregating, the fused EGFP is capable of folding and fluorescing brightly. In this screen, combinatorially randomized peptides were co-expressed in *E. coli* with the Aβ42-EGFP fusion protein. Peptides that resisted cellular degradation and prevented the aggregation of Aβ42 permitted EGFP to fold and fluoresce. Therefore, individual *E. coli* colonies expressing both a library peptide and Aβ42-EGFP were screened to select for those colonies that showed the greatest fluorescence.

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Abbreviations used: *EGFP, enhanced green fluorescent protein; ThT, Thioflavin T; HFIP, hexafluoroisopropanol; O.D.$_{600}$, optical density at 600 nm; IPTG, Isopropyl β-D-1-thiogalactopyranoside; PBS, phosphate buffered saline, pH 7.0.; HSP20, Heat Shock Protein 20.*

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Aβ42:          DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Library 1:                              MSNKGAIIVLMVVVVVIADSHS
                                              TTA   AAAAA
                                              NND   DDDDD
                                              SSG   GGGGG

Aβ42:          DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Library 2:                   MDKLVFFAEVVGSNK
                                  ALL   AA
                                  DII   DD
                                  GMM   GG
                                   VV
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Figure 1. Amino acid sequence of Aβ42 compared to the degenerate amino acid sequences of Library 1 and Library 2 peptides. Combinatorial mixtures are indicated with amino acids shown in bold. Underlined residues were conserved sequences designed to match Aβ42.

Materials and Methods

Materials

Synthetic peptides were prepared by GenScript Corporation. DNA purification kits were from Qiagen Inc. Klenow Fragment DNA polymerase and restriction enzymes were from New England Biolabs. Expand High Fidelity DNA Polymerase was from Roche. pET 28a and pCDF-1b plasmids were from Stratagene. DNA sequencing was performed by Davis Sequencing.

Construction of the Aβ42 Gene

The Aβ42 gene was constructed using polymerase chain reaction (PCR)-based gene assembly [33]. Ten single-stranded DNA oligonucleotides (Table S1, Supporting information) were designed to base-pair with their upstream and downstream pairing partners. Codons were optimized for expression in *E. coli*. An oligo master mix was prepared by adding 2 µl of a 100 µM solution of each oligonucleotide to a single tube. To assemble the full-length Aβ42 gene, 1 µl of the oligo master mix was PCR amplified using Expand High Fidelity Polymerase (Roche).

Construction of p Aβ42-EGFP

The synthetic Aβ42 gene was doubly digested with HindIII and EcoRI and ligated into an analogously digested p28EGFP plasmid that contains the gene for EGFP. [The EGFP gene, and other similar fluorescent proteins, is commercially available from Clontech Laboratories, Inc. The EGFP gene was PCR amplified using the primers 5′-GAA CTG GAC CAT ATG GTG AGC AAG GGC GAG GAG-3′ and 5′-GTT ACG CTG GAA TTC TTA CTT GTA CAG CTC GTC CAT GCC-3′ which produce an NdeI restriction site at the 5′ end of the gene and an EcoRI site at the 3′ end of the gene. The PCR product was doubly digested with NdeI and EcoRI restriction endonucleases (New England Biolabs) and ligated into an analogously digested pET28a plasmid (Novagen)]. The final construct, p28Aβ42-EGFP was verified with DNA sequencing. In this construct, the Aβ42 peptide is genetically linked to EGFP through a short flexible linker sequence. The Aβ42-EGFP gene was removed from the pET28a plasmid using NcoI and HindIII restriction endonucleases and ligated into an analogously digested pCDF-1b plasmid yielding pAβ42-EGFP.

Construction of the Peptide Libraries

Two peptide libraries were designed to match the suspected aggregation-prone regions of Aβ42. Combinatorial variation was introduced to the libraries using degenerate codons (Table S2, Supporting information). Library 1 was designed to associate with Aβ42 amino acids 29–42 and Library 2 was designed to associate with Aβ42 amino acids 17–21 (Figure 1). The two libraries were constructed using three synthetic DNA oligonucleotides (Table S2, Supporting information), as described in the supplementary materials.

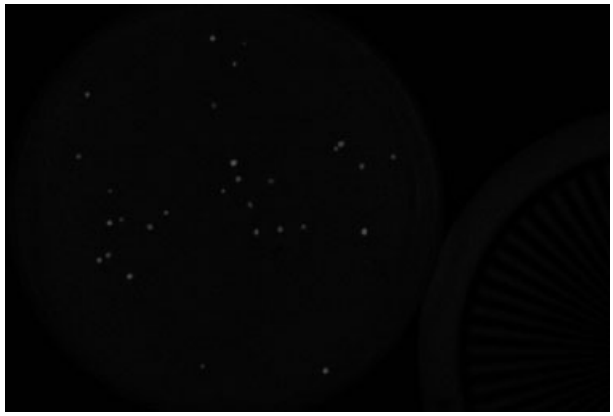

Figure 2. Colonies expressing both Aβ42-EGFP and library peptides are visualized under UV-light using a Bio-Rad Molecular Imager VersaDoc MP Imaging System. Colonies with the greatest level of fluorescence were selected for DNA sequencing and further *in vitro* tests. Note: The CCD camera detector does not show data in color. Data are shown as fluorescence emission intensity.

Screening Peptide Libraries

The pAβ42-EGFP plasmid (Strep^r) was cotransformed with either the Library 1 or the Library 2 plasmid library (Amp^r) into electrocompetent BL21 (DE3) *E. coli* cells (Stratagene) using a BTX ECM388 electroporator. The transformed colonies were plated on sterile nitrocellulose discs on LB media plates that contained both ampicillin and streptomycin. The plates were incubated for 15 h at 37 °C. The nitrocellulose discs, covered in individual colonies, were transferred to LB media plates containing ampicillin, streptomycin, and 2 mM IPTG. These plates were incubated at 37 °C for 3–6 h. Plates were scanned both visually and under 490 nm wavelength light (Figure 2) to select green-colored fluorescent colonies.

Quantification of Cell Culture Fluorescence

Selected colonies were grown to an $O.D._{600}$ of 0.7 before protein induction with 1 mM IPTG. The induced *E. coli* were incubated

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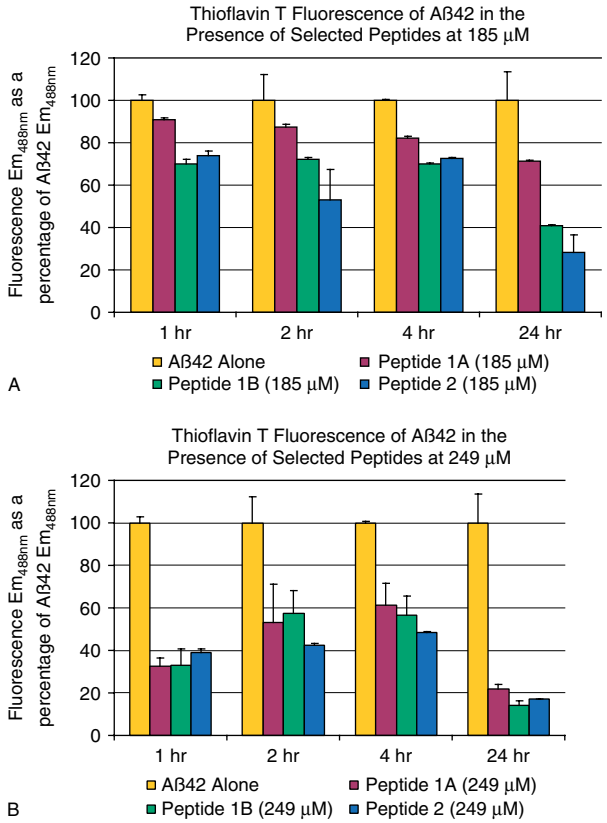

Figure 3. Time course of Aβ42 aggregation as monitored with ThT binding in the presence of selected peptides at (A) 185 and (B) 249 µM. Aβ42 was 40 µM for all samples. ThT fluorescence in the presence of each peptide is shown as a percentage of the ThT fluorescence of Aβ42 alone at each time point. The ThT fluorescence for Aβ42 alone increased for each time point. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

at 37 °C with shaking for 4 h. After 4 h, $O.D._{600}$ and fluorescence emission ($Ex_{490\ nm}$ and $Em_{516\ nm}$) of each culture was recorded. Only colonies showing an increase in fluorescence compared with cultures expressing Aβ42-EGFP alone were selected for further testing.

Preparing Disaggregated Aβ42

In 4.0 ml of HFIP, 0.5 mg synthetic Aβ42 (GenScript Corp) was dissolved and placed in a sonicating water bath for 20 min. The solution was divided into 400 µl aliquots and stored at −80 °C.

ThT Binding of Aβ42 in the Presence of Selected Peptide Inhibitors

ThT binding studies were performed as described by LeVine [34]. Disaggregated Aβ42 (as described above) was thawed and the HFIP removed over a stream of nitrogen gas. The resulting solid Aβ42 was dissolved in PBS buffer to yield a 0.2 mg/ml stock solution. This stock solution was divided evenly among tubes containing the selected peptides dissolved in PBS buffer. The in-solution concentration of Aβ42 peptide was 40 µM for each sample and the concentrations of selected peptides ranged from 1.2 mM to 20 µM. The samples containing Aβ42 and selected peptides were incubated at 37 °C with shaking (120 rpm). At various time points, 15 µl aliquots were removed and mixed with 485 µl of 3 µM

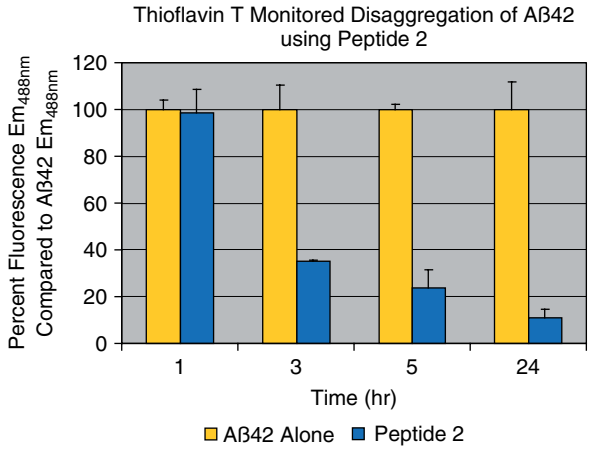

Figure 4. Disaggregation of preformed Aβ42 aggregates as monitored with ThT binding in the presence of Peptide 2. Peptide 2 (1.2 mM) showed substantial ability to disaggregate preformed fibrils (initial Aβ42 concentration was 40 µM). Data are the average of four separate trials conducted on separate days. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

ThT in 50 mM glycine buffer pH 8.5. The ThT mixture was incubated at room temperature in the dark for 15 min before recording the ThT fluorescence spectrum ($Ex_{450\ nm}$) using a Hitatchi F-7000 fluorescence spectrophotometer. ThT fluorescence ($Em_{488\ nm}$) in the presence of each peptide inhibitor was taken as a percentage of the ThT fluorescence of Aβ42 alone.

Monitoring the Disaggregation of Aβ42 with Selected Peptides

For 24 h, 0.2 mg/ml stock Aβ42 (described above) was incubated at 37 °C with shaking (120 rpm) to promote formation of Aβ42 fibrils. This resulting solution was evenly divided among tubes containing the peptide inhibitors at concentrations ranging from 10 µM to 1.2 mM. The preformed Aβ42 fibrils with the peptide inhibitors were incubated at 37 °C with shaking (120 rpm). At 1, 3, 5 and 24 h, 15 µl aliquots were removed and added to 485 µl of 3 µM ThT in 50 mM glycine buffer pH 8.5. The ThT mixture was incubated at room temperature in the dark for 15 min before recording the ThT fluorescence spectrum ($Ex_{450\ nm}$) using a Hitatchi F-7000 fluorescence spectrophotometer.

Results and Discussion

The 42-amino acid peptide, Aβ42, is highly amyloidogenic. The exact amino acids responsible for the self-aggregation of Aβ42 are not known, but it is believed that the two hydrophobic patches of Aβ42 may play a role. With that in mind, we constructed two peptide libraries designed to anneal to either of the two hydrophobic patches of Aβ42 (Figure 1). The degenerate peptide libraries were designed to maintain much of the hydrophobic character of the Aβ42 sequence. However, negatively charged aspartic acid residues were introduced into the combinatorial mix to act as potential aggregation breakers. The goal was to produce a peptide (or series of peptides) having a nonpolar face capable of annealing tightly to Aβ42, while displaying a highly charged and polar aspartic acid residue that could sterically block additional Aβ42 peptides from binding. The theoretical size of peptide Library 1 was 16 384 possible sequence variants while that of Library 2

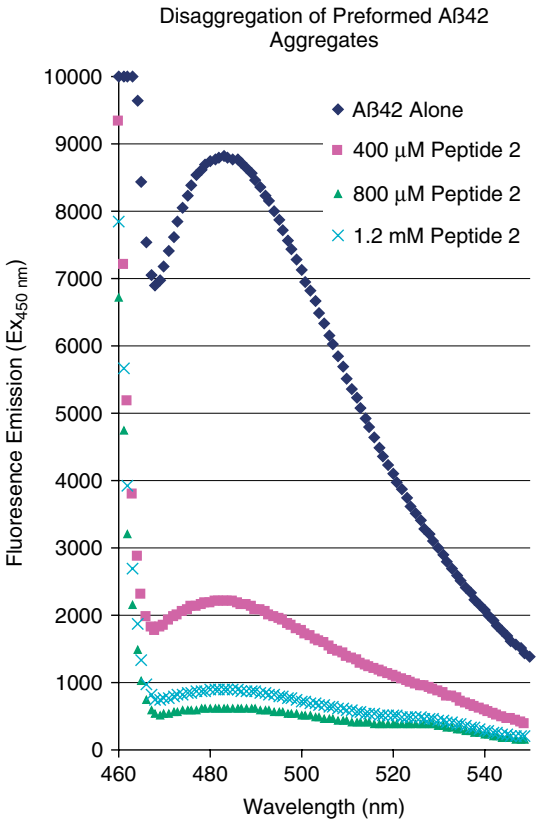

Figure 5. ThT fluorescence of preformed Aβ42 fibrils (40 µM) with varying concentrations of peptide 2. To promote aggregation, Aβ42 was incubated in PBS buffer for 24 h at 37 °C with vigorous shaking. Peptide 2 was added, at varying concentrations, to the preformed fibers and incubated for 24 h at 37 °C with vigorous shaking. After 24 h, the samples were tested with the ThT binding assay. The raw data are shown. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

was 1600. The actual number of clones generated was 55 000 for Library 1 and 35 000 for Library 2.

E. coli colonies co-expressing Aβ42-EGFP with library peptides were incubated on LB media plates containing IPTG. Colonies were irradiated with 490 nm light to visually identify those with the greatest amount of fluorescence emission at 516 nm (Figure 2). Six colonies were initially selected based on their fluorescence emission intensity. Of the six colonies selected, only three showed a substantially increased amount of fluorescence when grown in solution (Figure S1, Supporting information). Two of these colonies were selected from Library 1 (named peptides 1A and 1B) and the third colony was selected from Library 2 (named peptide 2). The plasmid DNA from these clones was purified and sequenced to identify the selected peptides (Table 1).

With any screen, it is important to verify the activity of the selected candidates using additional experimental tests. Candidates selected with this screen should be capable of inhibiting Aβ42 aggregation. However, this screen could unintentionally select for peptides that help EGFP to fold and fluoresce without necessarily preventing Aβ42 aggregation. To directly test the ability of the selected peptides to inhibit Aβ42 aggregation, the ThT fluorescence assay was used. ThT fluorescence has been shown to be a useful indicator for detecting and quantifying amyloid fibril formation [35,36]. ThT binds to the cross β-structure of amyloid proteins. The fluorescent properties of ThT change when the dye moves from an

Table 1. Selected peptide sequences

Peptide	Sequence
Peptide 1A	**M**SNKGA**S**IGLM**A**G**D**V**D**IADSHS
Peptide 1B	**M**SNKGA**SNA**LM**A**G**DGD**IADSHS
Peptide 2	**M**QKL**DVV**AED**A**GSNK

Peptides 1A, 1B, and 2 were selected for their ability to prevent Aβ42 aggregation as detected by an increase in Aβ42-EGFP fluorescence. Amino acids that differ from wild-type Aβ42 are shown in bold.

aqueous environment, to the aggregated amyloid protein. These fluorescent changes can be quantitated. As the concentration of amyloid increases, so too does the ThT fluorescence. The three selected peptides were commercially synthesized and dissolved in PBS buffer, pH 7.0. The aggregation time course of Aβ42 alone and in the presence of each of the selected peptides (at 185 and 249 µM) is shown in Figure 3. At 185 µM, peptide 1A shows modest ability to inhibit Aβ42 aggregation, while peptides 1B and 2 show strong inhibitory potential. At 249 µM, all three selected peptides show considerable inhibitory potential. Selected peptides incubated with ThT (in the absence of Aβ42) had the same fluorescence properties as ThT alone in buffer (data not shown).

While many substances have been shown to inhibit Aβ42 aggregation, few are known to disaggregate preformed Aβ42 fibers. Peptides 1A, 1B, and 2 were tested using ThT for their ability to disaggregate preformed Aβ42 fibers. Figure 4 shows the time course of Aβ42 disaggregation in the presence of peptide 2. While peptides 1A and 1B were found to inhibit Aβ42 aggregation, they were not found capable of disaggregating preformed Aβ42 fibers. However, peptide 2 was shown to inhibit Aβ42 aggregation as well as disaggregate preformed Aβ42 fibers. Concentrations of peptide 2 as low as 400 µM were found to disaggregate preformed Aβ42 (Figure 5) within 24 h. Peptide 2 is one of the few peptides we are aware of, along with those of Sato *et al*. and Soto *et al*., shown to disaggregate preformed Aβ42 fibers [26,27].

Conclusion

An Aβ42-EGFP construct was successfully used to select for peptides capable of inhibiting the aggregation of Aβ42. This screen selected for peptides that could resist *in vivo* degradation and inhibit Aβ42 aggregation. Two different peptide libraries, with a theoretical diversity of nearly 18 000 different sequences, were screened. From this screen, three peptides were ultimately selected for further investigation. ThT assays indicated that the selected peptides were strong inhibitors of Aβ42 aggregation. Subsequent testing demonstrated that peptide 2 is one of the few peptides known to disaggregate preformed Aβ42 fibers [26,27].

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Supporting information

Supporting information may be found in the online version of this article.

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