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Preparation and Screening of Catalytic Amyloid Assemblies

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Abstract

Aggregation of proteins into amyloids has long been recognized as one of the major contributors to disease and aging. Amyloids are known to catalyze their own formation but they have been considered the rock-bottom thermodynamic minimum of the protein fold without much functionality. We have recently demonstrated that aggregation of short peptides in the presence of metal ions gives rise to efficient catalytic activity. Here we present a detailed protocol for the synthesis and purification of these peptides and the preparation of amyloid-like fibrils. Then we describe an easy-to-perform, high-throughput assay to measure their hydrolytic activity.

Keywords

Self-assembly; Peptides; Amyloids; Catalysis; Hydrolysis

1. Introduction

Amyloid assemblies represent a highly stable, β -sheet rich arrangement of polypeptides. In many cases, the ability of proteins to misfold and aggregate into such assemblies has been implicated in aging and disease [1]. The polymeric (and potentially heterogeneous) nature of amyloids greatly hinders their studies, but it is becoming increasingly evident that even the simplest of amyloid arrangements can result in multiple structural forms, akin to the folding of soluble proteins [2]. Moreover, amyloids have been hypothesized to serve as primordial ancestors of modern proteins [3, 4]. If the polypeptide assemblies present during early stages of evolution had “protein-like” character, then it is possible that they were able to facilitate chemical transformations. Thus, we hypothesized that self-assembly of short, functionalized peptides into amyloid structures, held together through non-covalent interactions, would allow for creation of catalysts.

In order to test this hypothesis, we designed 7-residue peptides that can self-assemble into amyloid-like fibrils and are capable of enzyme-like catalysis in the presence of zinc cation (Table 1) [5]. Zn^{2+} stabilizes fibril formation and acts as a cofactor to catalyze ester hydrolysis. Reaction rate enhancements greater than a factor of 10,000 were observed after evaluating fewer than 10 designed peptides [5]. Moreover, the catalytic efficiencies of the fibrils are on par with those of some natural enzymes by weight. We also found that mixing

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¹²When acquiring change of absorbance per unit of time, fit only the linear portion of the data set to a straight line.

some of these peptides with different sequences resulted in a synergistic effect on their catalysis, providing an opportunity for high-throughput screening of multiple arrangements of functional groups in a single fibril. The catalytic activities of these self-assembling catalysts are impressive given the very small number of peptides screened, and this discovery opens new ways for designing bioinspired catalytic materials. We and others have already shown that this approach can be easily expanded to other reactions and peptide sequences [6, 7, 8, 9, 10, 11, 12, 13]. The ease with which we were able to discover catalysts supported by amyloid-like assemblies suggests that one should consider catalytic production of toxic species as one possible source of amyloid toxicity.

In this chapter, we present the synthesis and purification of peptides and their assembly into amyloid-like fibrils. We provide a detailed protocol for kinetic characterization of the hydrolytic activity of self-assembling peptides and peptide mixtures using *p*-nitrophenyl acetate (*p*NPA) as a substrate (Scheme 1). *p*NPA provides an established benchmark for hydrolytic reactions (e.g., ester, amide, lactone hydrolysis, and carbon dioxide hydration) and many enzymes are assayed using this reaction. Thus it allows for comparison with natural enzymes and designed protein catalysts. The kinetic assay is easy to perform, robust, and reproducible. It can be carried out in a high-throughput manner using inexpensive instrumentation that measures changes in UV-Vis absorbance.

2. Materials

Prepare all solutions using ultrapure water (Milli-Q water), analytical grade reagents, and HPLC grade organic solvents. Prepare and store all reagents at room temperature unless indicated otherwise. This protocol is optimized for pH 8, however it can be adopted for other conditions (see Notes 1, 3, and 13).

2.1. Peptide Synthesis and Purification

1. Dimethylformamide (DMF).
2. 5% Piperazine solution in DMF: Weigh out 12.85 g piperazine and dissolve it in 250 mL of DMF at 50 °C. Add 3.35 g hydroxybenzotriazole (HOBt) to the solution if the peptide sequence has glutamate and/or aspartate.
3. N,N-Diisopropylethylamine (DIEA).
4. [2-(6-Chloro-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate] (HCTU).
5. Methanol.
6. Rink amide MBHA resin.
7. Fmoc-protected amino acids.

¹To screen the peptide activity at different pH, use the following buffers: MES for pH 6–6.5, HEPES for pH 7–7.5, TAPS for pH 9–9.5, and CAPS for pH 10–10.5.

³Zinc precipitates as Zn(OH)₂ at higher pH. Lower the concentration of the zinc stock solution to 0.5 mM at pH 9 or higher. Always make this solution fresh, as precipitation may occur within hours.

¹³When working at different pH, use the following extinction coefficients of the product: 1700, 4300, 9100, 12,700, and 16,600 M⁻¹ cm⁻¹ at pH 6, 6.5, 7, 7.5, and 8, respectively. Use the extinction coefficient of 18,700 M⁻¹ cm⁻¹ at pH 8.5 and above.

8. Cleavage solution: Trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H₂O (95:2.5:2.5, v/v/v).
9. Nitrogen gas.
10. Ice-cold methyl *tert*-butyl ether.
11. Centrifuge.
12. Solvent A: 0.1% TFA in Milli-Q water.
13. Solvent B: 90% CH₃CN, 9.9% Milli-Q water, 0.1% TFA.
14. Reaction vessel.
15. Reverse-phase high performance liquid chromatography (HPLC) system.
16. C4 preparative column and C18 analytical column.
17. Matrix assisted laser desorption ionization instrument with a time of flight detector (MALDI-TOF).
18. Lyophilizer.

2.2. Peptide Stock Preparation and Kinetic Assay

1. Tris–HCl buffer: 1 M Tris–HCl, pH 8. Weigh out 4.85 g of Tris base and transfer to a 50 mL conical tube. Add 30 mL of water and adjust pH using HCl. Add water up to 40 mL. Readjust the pH if necessary (*see* Note 1).
2. ZnCl₂ solution: 50 mM ZnCl₂. Weigh out 1.70 g of anhydrous ZnCl₂, add 1 mL of 1 M HCl and 249 mL of water; mix the solution until ZnCl₂ dissolves (*see* Note 2).
3. Working buffer: 25 mM Tris, 1 mM ZnCl₂, pH 8. Measure about 30 mL of water into a 50 mL falcon tube, add 1 mL of 1 M Tris solution, and mix. Then add 0.80 mL of 50 mM ZnCl₂ and add water up to 40 mL (*see* Note 3).
4. 10 mM HCl (*see* Note 4).
5. Urea: 8 M urea. Weigh out 2.40 g of urea and add water up to 5 mL.
6. *p*-Nitrophenyl acetate (*p*NPA) stock solution: 100 mM *p*NPA. Weigh out 0.036 g of *p*NPA and dissolve it in 2 mL of acetonitrile. Store at 4 °C (*see* Note 5).
7. Isopropanol.
8. Acetonitrile.
9. 96-well plate (clear, flat bottom).
10. UV-Vis spectrophotometer.
11. Plate reader.

²The shelf life of this solution is at least 1 year.

⁴First make a 1 M HCl solution using concentrated HCl (12 M), then it can be further diluted to 10 mM.

⁵The shelf life of this solution is 1–2 months.

12. Multichannel pipette.

3. Methods

3.1. Peptide Synthesis and Purification

1. Weigh out 233 mg (0.1 mmol, 0.43 meq/g) of Rink-amide resin and place it into a reaction vessel. Swell the resin for 30 min in DMF at room temperature.
2. Remove the Fmoc group from the resin by adding 2 mL of the 5% piperazine solution for 5 min at 65 °C.
3. Wash the resin four times for 30 s each using DMF.
4. Activate the amino acid (0.3 mmol, 3 eq.) in a separate vial using 1 mL of HCTU (0.28 mmol, 2.8 eq.) in DMF and 104 µL of DIEA (0.6 mmol, 6 eq.).
5. Add the activated amino acid to the resin and let it couple for 7 min at 65 °C.
6. Repeat **steps 2–5** until the desired sequence is synthesized.
7. Acylate the N-terminus of the peptide by incubating it with a mixture of acetic anhydride (56.6 µL, 0.6 mmol, 6 eq.), DIEA (116 µL, 0.63 mmol, 6.3 eq.), and DMF (1 mL) for 5 min at room temperature.
8. Wash the resin twice for 30 s each using DMF.
9. Wash the resin with methanol twice for 1 min each.
10. Filter the resin and dry it under vacuum for at least 30 min.
11. Transfer the resin into a vial and cleave/deprotect the peptide by stirring the resin with 5 mL of TFA/TIS/H₂O (95:2.5:2.5, v/v) mixture for 2 h at room temperature.
12. Filter the solution through a glass wool and remove TFA by passing a gentle stream of nitrogen.
13. In order to separate protecting groups from the peptide, precipitate the peptide by adding 20 mL of dry ice cold methyl *tert*-butyl ether to the solution acquired in **Step 12**. Centrifuge the precipitate for 5 min at 1750 × *g* and decant the supernatant.
14. Wash the precipitate twice with 20 mL of dry ice cold methyl *tert*-butyl ether.
15. Dry the precipitate by passing a gentle stream of nitrogen.
16. Purify the peptide to at least 95% purity on an HPLC system with C4 preparative column by applying a linear gradient of solvent A and solvent B. Confirm peptide purity using HPLC with a C-18 analytical column and check the identity of peptides using MALDI-TOF.
17. Lyophilize pure peptide and store it at –20 °C until needed.

3.2. Preparation of Peptide Stock Solutions

1. Dissolve a small amount of solid peptide in 1 mL of 10 mM HCl (pH 2) (*see* Note 6). This solution is referred to as a pH 2 peptide stock.
2. Adjust the concentration of the pH 2 peptide stock to 1 mM. Use UV-Vis spectrophotometer to determine peptide concentration (*see* Note 7).
3. Prepare pH 8 stock solution by mixing 180 μ L of the pH 2 peptide stock with 20 μ L of isopropanol and 1.8 mL of the working buffer (*see* Note 8). The final concentration of peptide is 90 μ M.

3.3. Kinetic Assay

1. Turn on the plate reader and set up a kinetic reading protocol with the wavelength of 405 nm, 15 min scan time, and 30 s kinetic interval time at room temperature.
2. Pipette 50 μ L of the working buffer (blank) or 50 μ L of the pH 8 peptide stock solution (90 μ M peptide) into different wells on a 96-well plate.
3. Prepare *p*NPA substrate solutions with different concentrations of the substrate. Measure out ingredients shown in Table 2, immediately mix the solutions, and pour them into separate reagent reservoirs (*see* Notes 9 and 10).
4. Immediately add 150 μ L of the substrate solutions into the wells using a multichannel pipette and place the plate into the plate reader.
5. Start the kinetics reading.
6. Plot the acquired absorbance vs. time data and obtain change of absorbance per unit of time. To calculate initial rate (v_0), divide this number by the extinction coefficient of *p*-nitrophenol (16,600 $M^{-1} cm^{-1}$ at pH 8) (*see* Notes 11–13).

⁶Peptide stocks at pH 2 are stable for at least several weeks.

⁷Calculate the expected absorbance using Beer's law. In order to measure the absorbance of sample, dilute 10 μ L of the peptide stock solution using 10 mM HCl (the extent of dilution depends on the extinction coefficient of the peptide). Calculate the extinction coefficient of the peptides, using the following equation and the extinction coefficient of amino acids at 214 nm (Table 3) [14].

$$\epsilon_{\text{peptide}} (M^{-1} cm^{-1}) = \epsilon_{\text{peptide bond}} \times n_{\text{peptide bond}} + \sum_{i=1}^{20} \epsilon_{\text{aminoacid}(i)} \times n_{\text{aminoacid}(i)}$$

⁸Always make the pH 8 solution immediately before the experiment.

⁹ Zn^{2+} in the buffer solution also hydrolyzes *p*NPA, so *p*NPA stock solution should be the last component added to the substrate solutions.

¹⁰Other substrate concentrations can be used as long as the substrate is soluble in water (solubility is 2.9×10^{-3} M at 25 °C).

¹¹The path length of the absorbing solution in a well as measured by plate reader is not fixed and it depends on the volume of the solution, plate reader model, well dimensions, etc. Before taking kinetic measurements, calculate an empirical conversion coefficient: measure the absorbance of *p*-nitrophenol solution in your plates using plate reader and then in cuvette using UV-Vis spectrophotometer (1 cm path length). Use the following equation to calculate the conversion coefficient to normalize the observed absorbance to the 1 cm path length:

$$\text{Conversion coefficient} = \frac{A_{\text{UV-Vis}}}{A_{\text{plate reader}}}$$

$$v_0 = \frac{\Delta A}{t} / \epsilon$$

7. Account for the hydrolysis of substrate in buffer by subtracting initial rate in blank sample from the initial rate of peptide sample.
8. To obtain kinetic parameters (k_{cat} and K_M), plot the initial rate vs. substrate concentration and fit the acquired data to the Michaelis–Menten equation, where $[E]_0$ is the concentration of catalyst (*see* Note 14) and $[S]_0$ is the initial concentration of substrate at the onset of the reaction:

$$v_0 = \frac{k_{\text{cat}} [E]_0 [S]_0}{K_M + [S]_0}$$

To obtain k_{cat}/K_M values, fit the linear portion of the Michaelis–Menten plot to the following equation:

$$v_0 = \frac{k_{\text{cat}}}{K_M} [E]_0 [S]_0$$

3.4. Preparation of Peptide Mixtures

1. Prepare 4 mM peptide stock solutions in 10 mM HCl (pH 2 stock) following the instructions described in Subheading 3.2.
2. Mix two peptide stock solutions in different percent ratios (0, 8, 16, 24, 32, 40, 48, 55, 70, 85, and 100%) to produce 100 μL samples.
3. Add 300 μL of 8 M urea to each of the 100 μL peptide samples and let the mixture sit for 15 min (*see* Note 15).
4. Add 3.6 mL of working buffer into this solution and mix well by inverting the tube several times. The resulting is a pH 8 peptide stock with a final peptide concentration of 100 μM . Mixing of the already formed fibrils by pipetting should be avoided to ensure reproducibility as the fibrils tend to stick to plasticware.
5. Pipette 50 μL of working buffer (blank) or 50 μL of the pH 8 peptide solution (100 μM) into different wells on a 96-well plate (*see* Note 16).
6. Follow **steps 3–8** described in Subheading 3.3 to perform the kinetics assay on these peptide samples.

¹⁴The final concentration of peptide is 22.5 μM , however concentration of catalyst is 11.25 μM because two peptides coordinate one zinc ion to form one catalytic unit.

¹⁵It is important to mix the peptide stocks at pH 2 in the presence of urea. Urea precludes peptide association and ensures effective mixing at the molecular level.

¹⁶For the blank, mix 100 μL of 10 mM HCl, 300 μL of 8 M urea, and 3.6 mL of working buffer.

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Scheme 1.
Hydrolysis of *p*-nitrophenylacetate

Table 1Esterase activity of designed peptides at pH 8 in the presence of 1 mM Zn²⁺

Peptide	Sequence	$k_{\text{cat}}/K_{\text{M}}(\text{M}^{-1} \text{s}^{-1})$
1	Ac-LHLHLQL-CONH ₂	30 ± 3
2	Ac-LHLHLYL-CONH ₂	13 ± 5
3	Ac-LHLHLKL-CONH ₂	12 ± 2
4	Ac-LHLHLRL-CONH ₂	18 ± 4
5	Ac-IHIHRI-CONH ₂	22 ± 8
6	Ac-VHVHVRV-CONH ₂	26 ± 4
7	Ac-IHIHIQI-CONH ₂	62 ± 2
8	Ac-VHVHVQV-CONH ₂	32 ± 2

Table 2Preparation of *p*NPA substrate solutions

	<i>V</i> (Tris working buffer), mL	<i>V</i> (acetonitrile), μ L	<i>V</i> (100 mM <i>p</i> NPA stock solution), μ L	Final [<i>p</i> NPA] in the reaction mixture, μ M
Solution 1	4.9	87	13	195
Solution 2	4.9	75	25	375
Solution 3	4.9	62	38	570
Solution 4	4.9	50	50	750

Table 3

Extinction coefficients of amino acids at 214 nm

Amino acid	Extinction coefficient at 214 nm ($M^{-1} \text{ cm}^{-1}$)
A	32
R	102
N	136
D	58
C	225
Q	142
E	78
G	21
H	5,125
I	45
L	45
K	41
M	980
F	5,200
P	23,675 (or 30 if at N3-terminus)
S	34
T	41
W	29,050
Y	5,375
V	43
Peptide bond	923