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Proteinase K-catalyzed synthesis of linear and star oligo-L-phenylalanine conjugates

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ABSTRACT

Chemo-enzymatic synthesis of peptides is a green/clean chemical reaction that offers high yields without using organic synthesis, and serves as an alternative to traditional peptide synthesis methods. This report describes the chemo-enzymatic synthesis of oligo-L-phenylalanine mediated by proteinase K from *Tritirachium album*, which is one of the most widely used proteases in molecular biological studies. The synthesized linear oligo-phenylalanine showed a unique self-assembly in aqueous solutions. To further functionalize linear oligo-L-phenylalanine as a low-molecular-weight gelator, it was co-synthesized with tris(2-aminoethyl)amine to obtain star-oligo-L-phenylalanine, which was bioconjugated to demonstrate its self-assembly into fluorescent fibers. The self-assembled fibers of star-oligo-L-phenylalanine formed fibrous networks with various branching ratios, which depended on the molecular weights and molecular aspect ratios of star-oligo-L-phenylalanine. This is the first study to demonstrate that proteinase K is a suitable enzyme for chemo-enzymatic co-synthesis of oligopeptides and star-shaped hetero-peptides.

KEYWORDS. Chemo-enzymatic synthesis; oligo-phenylalanine; proteinase K; star peptide; tris(2-aminoethyl)amine.

Introduction

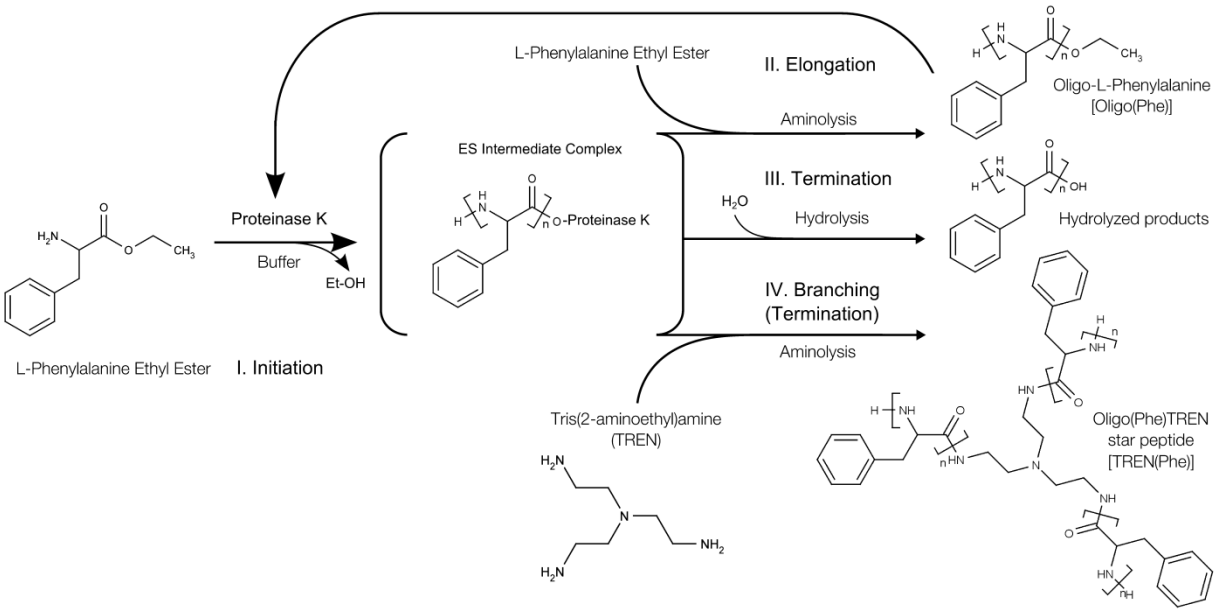
Peptides are an emerging class of functional biopolymers with unique chemical, physical, and biological properties that are not demonstrated by synthetic polymers. Peptides are short linear chains of amino acids linked together in either repetitive or non-repetitive motifs. Among several technologies used for peptide synthesis, solid phase and recombinant syntheses are the most commonly used.[1, 2] In solid-phase peptide synthesis, protected amino acid are bonded to other amino acids through a process that requires multiple steps of protecting and de-protecting the carboxyl and amine groups of amino acids; nevertheless, this technique can control the composition and length of the peptides being synthesized. However, a lack of stereoselectivity and the use of strongly polluting agents are limitations of the method.[3] Recombinant expression allows peptide synthesis to be scaled up easily, but microorganisms often poorly express repetitive peptides,[2] especially cationic or hydrophobic peptides.[4]

An alternative approach is chemo-enzymatic synthesis,[1, 2] wherein an enzyme is used as a catalyst to produce peptide bonds. Enzymes enable selective reactions to occur because their active sites are stereoselective and have specific affinities for substrates.[5] Proteases (peptidase) are one of the most studied enzymes for peptide synthesis because they can act on the polypeptide backbone.[6] Peptides can be synthesized chemo-enzymatically using 2 approaches. The first approach is thermodynamically controlled synthesis (TCS), which represents the reverse reaction of hydrolysis. Proteases are enzymes that hydrolyze peptide bonds, but the hydrolysis reaction can be reversed.[1, 2] The reverse reaction is induced most conveniently by using organic solvents[6] that increase the solubility of products and limits hydrolysis, but an excess of organic co-solvents can inactivate enzymes.[6, 7] Because the reversal of hydrolysis with organic solvents is carried out in absence of water or with as little water as possible,[7] the reaction

equilibrium is changed.[1] However, any type of protease can be used in TCS because the protease serves as a mold with affinity for the substrate and reduces the energy needed for the process.[2] TCS requires large amounts of enzymes, but the yields are lower than those with other alternatives[6] such as kinetically controlled synthesis (KCS).[1,2,5,6] In the case of KCS, activated amino acids are used, often with a modified ethyl or methyl ester as the carboxylic end. After the first enzymatic step, which is similar to esterase activity, the enzyme forms a covalent intermediate complex with the substrate (ES complex. Scheme I), allowing the amine group of the other amino acid to act as a nucleophile. The enzyme functions as a transferase, and after aminolysis has generated a peptide bond, the product is released. If water acts as a nucleophile, hydrolysis occurs and terminates the reaction. Because a covalent ES complex must be formed, only serine and cysteine proteases can be used for this strategy.[1] The proteases used most commonly in chemo-enzymatic synthesis of polypeptide are papain, bromelain, and chymotrypsin.[2,5,8] Unlike with TCS, with KCS the yield and efficiency of synthesis are affected by the type of enzyme, or conditions of synthesis,[1,9] indicating that the search for new enzymes for use as catalysts is critical for developing chemo-enzymatic synthesis of polypeptides.

Proteinase K (pro K) (EC 3.4.21.64) from *Tritirachium album* is one of the most widely used proteases in molecular biology.[10] This serine protease works in broad pH and temperature ranges, being able to operate on substrates and under conditions not suitable for other enzymes.[11] Pro K shows affinity for aromatic or hydrophobic residues, and its hydrolytic activity is promoted strongly by long N-terminal regions of peptide substrates.[11] Pro K can hydrolyze numerous peptide substrates, and even some non-proteins such as Poly(l-lactic acid),[12] which made us consider that peptides can be co-synthesized with non-amino acids using pro K.[13] These characteristics make pro K an interesting candidate for the synthesis of star (branched) aromatic

peptides, which can function as low-molecular-weight gelators (LMWGs). LMWGs are small molecules that can self-assemble into gels or nanostructures through hydrogen bonding, hydrophobic interactions, and π–π stacking in aqueous solutions.[14] Phenylalanine-based peptides have been widely studied as key LMWGs [14, 15, 16, 17, 18] because these peptides can form nanostructures or gel-like molecular networks with distinct characteristics depending on the synthesis conditions.



Scheme I. Proteinase K-catalyzed oligo-l-phenylalanine synthesis via kinetically controlled synthesis (KCS)

This report describes KCS of oligo-L-phenylalanine [oligo(Phe)] in aqueous solution mediated by pro K. The resultant product self-assembled in amyloid-like fibers, which also form a gel-like fibril network. The self-assembly behavior was controlled by peptide concentration and temperature. To functionalize oligo(Phe) by branching the peptide, it was co-synthesized with Tris(2-aminoethyl)amine (TREN), whose amines acted as nucleophiles (Scheme I). This organic ligand, used traditionally in coordination chemistry, can increase the flexibility and alter the

morphology of peptide structures.[19] Oligo(Phe)-TREN conjugates [TREN(Phe)] self-assembled into fluorescent fibers (Scheme I). We demonstrate that the star structure of peptides functionalizes LMWGs[20] and that pro K is a suitable enzyme for synthesizing peptides precisely using organic ligands.

EXPERIMENTAL SECTION

Materials: L-phenylalanine ethyl ester hydrochloride (L-Phe-Et), L-phenylalanine methyl ester hydrochloride (L-Phe-Met), and proteinase K (pro K) were purchased from Wako Pure Chemical Industries (Osaka, Japan). L-Phenylalanine (PheOH), tris(2-aminoethyl) amine (TREN) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). Standard chemicals were purchased from Wako Chemical Co. (Kanagawa, Japan).

Proteinase K-Catalyzed Oligo(Phe) Synthesis. Synthesis conditions were determined based on previous protocols [8, 9, 21, 22]. Chemoenzymatic reaction at pH 8 is described as a typical example. Standard reactions were carried out with a final volume of 5 mL, 0.6 M L-Phe-Et, 1 mg/mL Pro K (21 U/mg) in 0.9 M phosphate buffer in a 25 mL glass reaction tube. The reactions were performed with stirring (1000 rpm) in EYELA ChemiStation (Tokyo, Japan) at 40 °C for 3 h. In order to optimize L-Phe-Et ester polymerization conditions with pro K, reactions were carried out by triplicate, varying the amount of L-Phe-Et (0 to 1 M), concentration of enzyme (0 to 10 mg/mL, 0 to 210 U/mg), temperature (24 to 60 °C) and pH (6 to 10). Citrate buffer (pH 6; 0.9 M), phosphate buffer (pH 8; 0.9 M), Tris(hydroxymethyl)aminomethane-HCl buffer (TRIS) (pH 8; 0.9 M) and sodium carbonate buffer (pH 10; 0.9 M) were used for each reactions at different pH. After the reaction, for inactivation of the enzyme, the reaction mixture was heated at 100 °C for 10 min and cooled to 25 °C. Oligo(Phe) was purified by centrifugation at 12,000 *g*,

25 °C for 5 min, pellets were suspended in 10 mL Milli-Q (MQ) water. The resultant solution was then dialyzed overnight against MQ water using cellulose dialysis membranes (Spectra/Por® Biotech cellulose ester (CE); MWCO. 100-500 Da; Spectrum Laboratories Inc., Rancho Dominguez, CA). The oligo(Phe) solution was frozen at -80 °C and lyophilized.

Also, a time course study was performed on the pro K-mediated oligo(Phe) synthesis. The reactions were carried out at 40 °C and were stopped by addition of 1 mL of chilled proteomic grade acetone at different time points (0 to 180 min). Following, the reaction mixture was centrifuged at 16,000 *g*, 25 °C for 5 min. The resultant pellet was dried at 60 °C for a week. Based on the weight of the dried product, yield of oligo(Phe) in the reaction was calculated.

Synthesis of TREN Based Peptides. Tris(2-aminoethyl) amine (TREN) was used as nucleophile during the aminolysis extension step in the kinetically controlled peptide reaction (Scheme I). Several ratios of monomer (L-Phe-Et):TREN were assayed (1:6, 1:9, 1:15 and 1:30) in 0.9 M phosphate buffer (pH 8) with 1 mg/mL of pro K. The reactions were performed as described above in oligo(Phe) synthesis.

Nuclear Magnetic Resonance. ^{1}H NMR, gCOSY (Two Dimensional J-correlation spectroscopy with gradient coherence selection) and gHMBCAD (Two dimensional heteronuclear multiple-bond J-correlation spectroscopy with adiabatic 180° X-nuclei pulses and gradient coherence selection) spectra were recorded on Varian NMR System 500 (500 MHz) spectrometer (Varian Medical Systems, Palo Alto, CA) at 25 °C controlled with VnmrJ. The lyophilized samples were suspended in deuterated dimethyl sulfoxide (DMSOd6) containing 1% Trifluoroacetic acid (TFA) at 10 mg/mL; 256 scans were recorded. Tetramethylsilane (TMS) was used as internal reference at 0.00 ppm. Data were processed and analyzed by ACD/NMR

Processor Academic Edition, version 12.01 (Advanced Chemistry Development, Inc., Toronto, On, Canada, www.acdlabs.com, 2010).

Tris(2-aminoethyl) amine: ^{1}H NMR (500 MHz, DMSO-d_6 : 1% CF_3COOH, TMS δ ppm): 2.48 (t, J=5.87 Hz, 6 H); 2.67 (t, J=5.99 Hz, 6 H); 4.25 (s, 9 H).

Oligo(Phe): ^{1}H NMR (500 MHz, DMSO-d_6 : 1% CF_3COOH, TMS δ ppm): 1.08 (t, *J*=7.09 Hz, 3 H) (**a**); 3.02 - 3.24 (m, 28 H) (**b**); 4.05 - 4.13 (m, 2 H) (**c**); 4.16 (t, *J*=6.97 Hz, 12 H) (**d**); 4.24 (t, *J*=6.72 Hz, 1 H) (**d'**); 7.10 - 7.45 (m, 66 H) (**e**); 8.44 (br. s.) (**f**); 8.67 (br. s.) (**f'**).

TREN(Phe) 1:6: ^{1}H NMR (500 MHz, DMSO-d_6 : 1% CF_3COOH, TMS δ ppm): 2.64 (t, J=5.87 Hz, 6 H) (**t1**); 2.92 - 3.00 (m, 6 H) (**t2**); 3.11 (d, J=6.36 Hz, 16 H) (**b**); 4.11 - 4.24 (m, 8 H) (**d**); 7.22 - 7.45 (m, 42 H) (**e**); 7.97 (br. s.) (**t3**); 8.38 (br. s.) (**f**).

TREN(Phe) 1:30: ^{1}H NMR (500 MHz, DMSO-d_6 : 1% CF_3COOH, TMS δ ppm): 1.08 (t, J=7.09 Hz, 1 H) (**a**); 2.61 - 2.68 (m, 6 H) (**t1**); 2.98 (br. s., 6 H) (**t2**); 3.12 (d, J=6.60 Hz, 45 H) (**b**); 4.14 (t, J=6.36 Hz, 21 H) (**d**); 7.11 - 7.52 (m, 119 H) (**e**); 8.08 (br. s.) (**t3**); 8.39 (br. s.) (**f**).

Fourier Transform Infrared spectroscopy (FTIR). FTIR absorption spectra of lyophilized samples [oligo(Phe) and Phe(TREN)] were collected using a Shimadzu IR- Prestige-21 FTIR spectrometer (Shimadzu, Kyoto, Japan) equipped with a multiple-reflection, horizontal MIRacle ATR attachment from 700 to 2000 cm^{-1} at 4 cm^{-1} resolution, using 100 scans.[21]

Optical and Confocal Laser Scanning Microscopy. Self-assembling and fiber formation were monitored by optical microscopy, in these experiments 15 μL of enzymatic reaction or peptide solution (10 mg/mL) were transferred onto a cover slip. Additionally, fixed samples were

performed as described in literature[24], briefly, peptide samples were dissolved in 60% methanol in water, 10 μL drop of solution was loaded onto a glass slide and dried at 25 °C. The samples were visualized in a polarized light microscopy (BX51, Olympus, Hamburg, Germany). Confocal laser scanning microscopy was performed in a LSM 700 (Carl Zeiss Jena, Germany) with an excitation wavelength of 405 nm and 450 nm of emission.

Scanning Electron Microscopy. Samples were dissolved at 10 mg/ml in 60% methanol, 10 μL drop of solution was dried in freshly cleaved mica and coated with gold. SEM images were recorded using a JSM JEOL 6330F scanning electron microscope (JEOL, Tokyo, Japan) operating at 7 kV.

Cell cytotoxicity experiments. Human Embryonic Kidney 293 cells (HEK293) (8×10^4 cells/mL) were cultured in 96-well micro plates (100 μL/well) and incubated overnight at 37 °C in Dulbecco's modified Eagle's medium (D-MEM) without phenol red (Wako Chemical Co.), supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 ng/mL basic fibroblast growth factor, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL of amphotericin B. Stock solutions of oligo(Phe), Phe(TREN), PheOH dissolved in Dulbecco's phosphate buffer saline [D-PBS(-)] (pH 7.4) were added to each well with a final concentration 0.5 to 50 mM. Control reactions were performed with addition of D-PBS(-). Following incubation for 8 hours at 37 °C, cell viability was evaluated using CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI). The cell viability was calculated as follows: [absorbance at 490 nm of the cell culture incubated with sample]/[absorbance at 490 nm of the negative control] x 100. Each experiment was repeated 3 times.

Results and Discussion

Although pro K is widely used in molecular biology, to our knowledge this is the first time pro K has been studied for KCS. Optimal conditions for peptide synthesis must be determined to enhance product yield and to increase the average degree of polymerization (DP_{avg}).[21] Previously, oligo(Phe) was synthesized successfully using bromelain[22] (16.6 mg/mL, 125 mM L-phenylalanine ethyl ester (L-Phe-Et), 0.25 M phosphate buffer, 30% methanol, pH 8, 40 °C, 3 h; yield 51%, DP_{avg} 8.2) and papain[23] (12 mg/mL, 200 mM L-phenylalanine methyl ester (L-Phe-Met), 1 M phosphate buffer, pH 7, 40 °C, 24 h; yield 60%, DP_{avg}. 6). Pro K-mediated synthesis of oligo(Phe) here followed KCS (Scheme I). Organic solvents, which are not required for KCS, were not used in reactions; this was in line with the concept of clean technology, which is one of the main advantages of enzymatic peptide synthesis over solid-phase peptide synthesis.

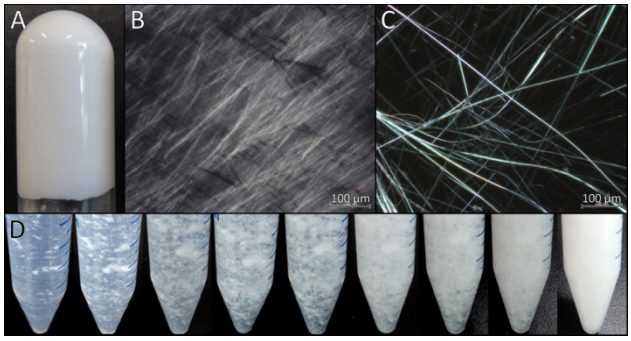


Figure 1. Photograph of oligo-phenylalanine synthesized after 3 h (A). Optical microscopy images of fibers in water (B) and under polarized light (C); scale bars represent 100 μm. Oligo-phenylalanine synthesized using proteinase K in 1 h; time-lapse series interval, 10 min (D).

Effect of pH and temperature in oligo(Phe) synthesis. To clarify the effect of pH on synthesis, reactions were carried out in 0.9 M of these buffers: sodium carbonate (pH 10), sodium phosphate and TRIS-HCl (pH 8), and sodium citrate (pH 6). After incubating for 1 h, the

reaction mixtures appeared to change, forming a precipitate in the bottom of the tubes that transformed into a gel-like structure eventually (Figure 1). Negative control samples without pro K showed no detectable changes, indicating that the precipitation was caused by the enzymatic reaction. The highest yield was achieved at pH 8 (Figure 2A), and the yield of reactions in phosphate buffer (65.5% ± 4.2%) was higher than in TRIS buffer (50.5% ± 3.3%). In KCS, yield and DP_{avg} are affected by the reaction pH,[21] because pH affects enzymatic activity and product solubility concomitantly. Solubility of phenylalanine peptides depends strongly on their self-assembling properties.[24] For example, diphenylalanine (Phe-Phe) starts self-assembling at its isoelectric point (pH 5.5).[25] Because the optimal pH of reactions tends to decrease in KCS,[26] alkaline conditions delay the precipitation or self-assembly of products. Figure 2 shows yields at distinct pH values and temperatures. At pH 10, reaction yields were lower and hydrolysis was increased (Figure 2A). Similarly, although increasing the temperature improved product solubility, the yield was diminished and hydrolysis was increased (Figure 2B). The yield and DP_{avg} at 24 °C and 40 °C were not significantly different, but the reaction kinetic was more stable at 40 °C (Figure 2B). Based on these results, phosphate buffer (pH 8) at 40 °C was chosen for further optimization studies.

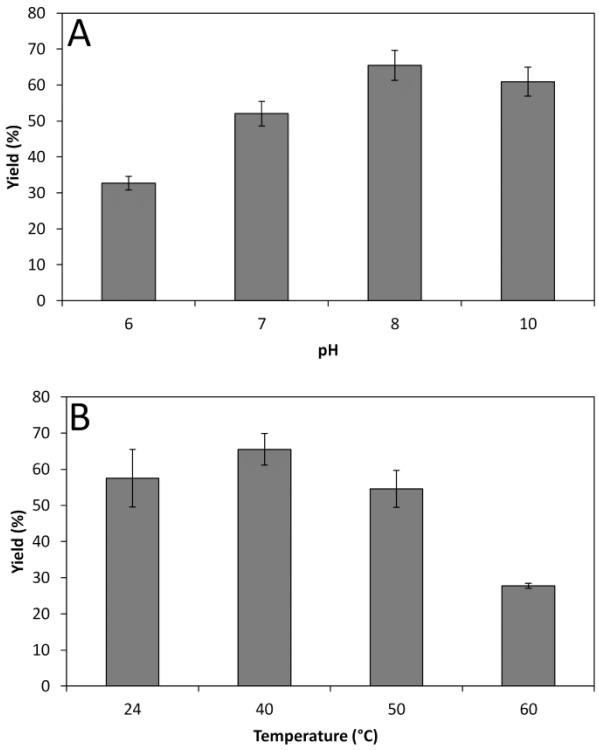


Figure 2. Effect of pH (**A**) and temperature (**B**) on the yield of oligo-phenylalanine from 0.6 M L-phenylalanine ethyl ester using 1 mg/mL of proteinase K. Error bars represent the standard deviation of the replicates.

Effect of enzyme and substrate concentrations on oligo(Phe) synthesis. Unlike in TCS, in KCS, enzyme/substrate ratios must be finely tuned to maximize product yield.[1, 5, 9] Thus, reactions with several concentrations of pro K were examined (Figure 3A), which showed that precipitates formed more quickly in reactions with higher concentrations of enzyme. Additionally, when reaction yields were measured at 3 h and 8 h, the highest concentrations of enzyme (5 and 10 mg/mL) were found to produce lower yields and hydrolyze the product (loss of ester end) (Figure 3A). Pro K appears to be more active than other proteases for KCS of peptides[22, 23] because a 10-times lower concentration of the enzyme was required for oligo(Phe) synthesis. However, pro K can hydrolyze products when used at high concentrations, especially

with lengthy incubation times (Figure 3). The kinetics of the reaction using 0.6 M of L-Phe-Et and 1 mg/mL of pro K demonstrated that 120 min was the optimal reaction time to achieve the maximal yield of approximately 65% (Figure 3B). The reaction behavior was similar to that reported for bromelain-mediated synthesis of oligo-phenylalanine,[22] but with pro K the yield was higher and the reaction time was shorter than with bromelain. Moreover, bromelain required 20% methanol with phosphate buffer for the reaction.[22] The effect of substrate concentration was also studied using 1 mg/mL pro K in reactions (Figure 3C), and the highest yield was obtained with 0.6 M of L-Phe-Et. Perhaps because of pro K's strong hydrolytic activity, a higher concentration of monomer is needed to induce KCS of oligo(Phe) with pro K than with bromelain[22] (0.125 M) or papain[23] (0.2 M), but productivity is better in terms of time and reaction volume. Moreover, by using pro K, product purity can be enhanced, because a lesser amount of the enzyme is required for synthesis.

Effect of monomer type on oligo(Phe) synthesis. Although the substrate affinity of pro K is not fully known, the enzyme shows distinct affinities for substrate in its esterase activity.[11] To study pro K's substrate specificity for monomers in KCS, the yields of L-Phe-Et and L-Phe-Met reactions were evaluated. Reactions were carried out under the optimal conditions defined above, 0.6 M of monomer, 1 mg/mL of pro K, 0.9 M phosphate buffer pH 8, for 3 h. L-Phe-Met and L-Phe-Et reactions showed yields of 67.63% ± 4.18% and 65.48% ± 4.15%, respectively, although structural analysis showed that in the L-Phe-Met reactions, approximately 70% of products were hydrolyzed after 3 h. L-Phe-Met has been reported to hydrolyze products,[23] but the extent of hydrolysis was not substantial. Thus, with pro K as a catalyst, distinct products (OH or ester end) can be synthesized by selecting methyl or ethyl esters monomers.

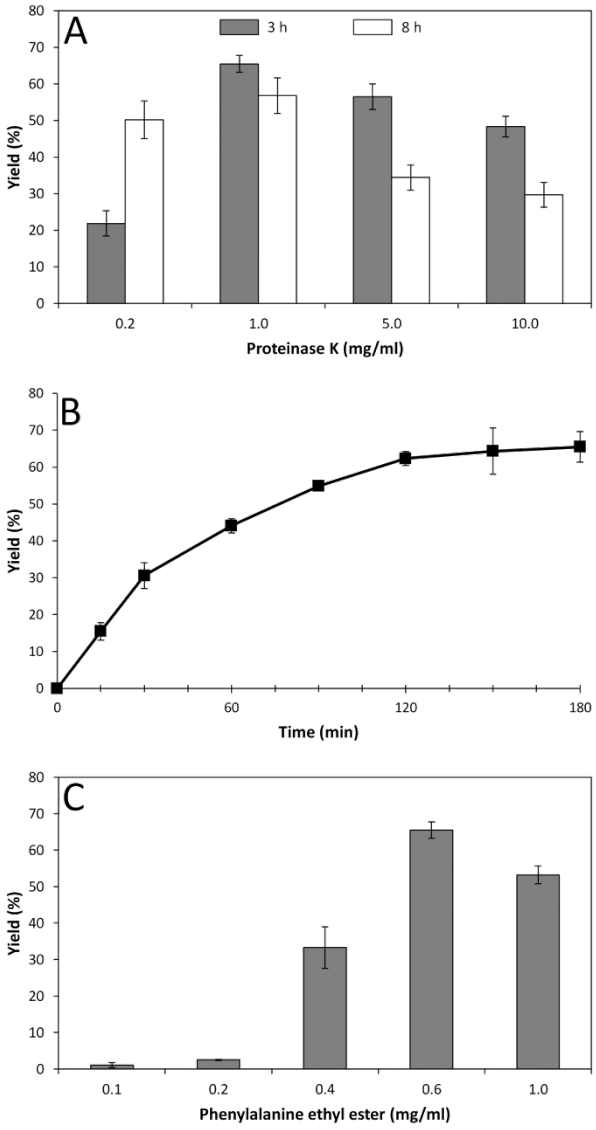


Figure 3. A. Effect of the concentration of proteinase K on the reaction yield at 3 h and 8 h. B. Time course of reaction using 0.6 M L-phenylalanine ethyl ester in 0.9 M phosphate buffer, pH 8, containing 1 mg/mL of proteinase K. C. Effect of monomer concentration in the synthesis of oligo-phenylalanine by proteinase K. Error bars represent the standard deviation of replicates.

Molecular weight and chemical structure of oligo(Phe). NMR signals were assigned according to references[22] and 2D NMR (gCOSY) (Figure S1A). DP_{avg} was calculated using the relative integration values of N-terminal CHα (d') and ethyl protons (c and a) to internal chain

CHα (d), CHβ (b), and the phenyl ring (e).[22] Subsequently, the degree of hydrolysis (loss of ester end) was calculated using the relative integration values of d' against c and a, and the carboxylic signals in carbon-proton long-range interactions (gHMBCAD) (Figure S1). The DP_{avg} was 12 ± 0.5 after 3 h for reactions carried out using 0.6 M L-Phe-Et, 1 mg/mL of pro K in phosphate buffer (pH 8) at 40 °C. Reactions carried out in phosphate buffer with 0.6 M of L-Phe-Met showed a DP_{avg} of 6.9. Because the L-Phe-Met sample contained a high proportion of hydrolyzed products as noted above (Scheme I), to avoid overestimating DP_{avg}, in addition to accounting for methyl and terminal groups, the ratio of the integrated areas of amino ends and chain repetition must be calculated. The yield obtained was higher than that reported by Schwab *et al.*,[23] although with similar DP_{avg}. In contrast, the reactions carried out in TRIS buffer did not show substantial hydrolysis, with DP_{avg} values of 4.8 and 4.7 for L-Phe-Met and L-Phe-Et synthesis, respectively. The TRIS buffer appears to limit the solubility of oligo(Phe) and thereby reduce hydrolysis and DP_{avg}. The NMR studies of purified product did not show the contamination of protease, suggesting that the concentration of pro K in purified product was low enough to be ignored when properties of oligo(Phe) and TREN(Phe) were discussed. Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry was not used to determine the molecular weight of oligo(Phe), because this method was reported to detect its fragmented products rather than oligo(Phe), resulting in underestimation of the molecular weight.[22]

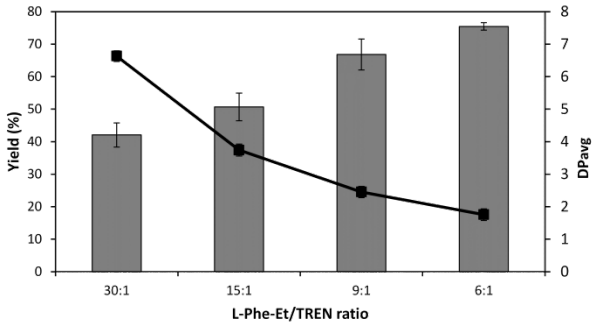


Figure 4. Bars: yields of oligo-phenylalanine:Tris(2-aminoethyl) amine [TREN(Phe)] polymers with synthesis carried out in 0.9 M phosphate buffer pH 8 with 1 mg/mL of proteinase K. Line: Average degree of polymerization of polymers (Dp_{avg}). Error bars represents the standard deviation of replicates.

Synthesis and structural analysis of TREN-based oligo(Phe). TREN is an organic ligand that can increase peptide flexibility and, when combined with LMWGs such as oligo(Phe), produce star peptides with new properties.[19, 20] Recently, chemically synthesized Phe-Phe TREN conjugates were reported to self-assemble into structures with diverse morphologies.[19] $(Phe\text{-}Phe)_3$TREN conjugates were synthesized through several steps using protected amino acids and strongly polluting agents,[19] and these star peptides self-assembled into branched fibers and spheres.[19] Because pro K can act not only on peptides substrates but also polyesters, we sought to use KCS-mediated chemo-enzymatic synthesis with pro K to produce star peptides. TREN-based oligo-Phe synthesis was designed as a co-polymerization process using the conditions optimized for linear polymers. In this process, one of the 3 free amino groups of TREN acts as a nucleophile (Scheme I) for the L-Phe-pro K complex, producing a star peptide. Similarly, free amines of TREN(Phe) act as nucleophiles until the size or molecular interactions changes the solubility. To characterize the mechanism of this reaction, several ratios of TREN and monomer were used (TREN:monomer, 1:6, 1:9, 1:15, 1:30), and reaction yields were found to range from 42% to 75% (Figure 4).

DP_{avg} values of TREN(Phe) peptides were calculated by accounting for the integration values of t1 and t2 signals as 6 H (Experimental Section). The resulting values were divided into the theoretical tri-branch points. As a result, the distinct ratios used in TREN(Phe) synthesis (1:6, 1:9, 1:15, 1:30) showed that DP_{avg} values of peptides decreased in relation to the amount of TREN

(Figure 4). By using TREN, polydispersity of the reaction can be lowered. In the sample with 1:30 ratio, linear and star polymer (methyl signal a) co-existed, suggesting that higher ratios do not enhance the DP_{avg} of peptides. NMR signals (Figure S2) were assigned as described[19, 22, 27] and according to the study of oligo(Phe) (Figure S1). The shift in TREN signals observed were similar to that reported for TREN conjugates.[19, 27] In gCOSY analysis, the amino signal at 7.89 ppm (t3) was found to correlate with a signal at 2.94 ppm (t2), which in turn is correlated with 2.64 ppm (t1). Additionally, gHMBCAD connected TREN and phenylalanine signals (Figure S2), and the connection between TREN and oligo(Phe) was confirmed using Fourier transform infrared spectroscopy (Figure S3), which revealed a peak of 1643 cm^{-1} that is not present in oligo(Phe) and was reported for the NCO bond in TREN conjugates.[19, 27]

Microscopic observation of oligo(Phe) and TREN(Phe) structures

Oligo(Phe) reactions acquired fibrous scaffold appearance after reaching 40% yield (1 h in standard reaction) (Figures 1D and 3B). Microscopic observation of reaction mixtures showed that the gel was composed of long birefringent fibrils with a diameter of 1 μm (Figure 1). Because self-assembly of phenylalanine peptides[24] is affected by the solubility of peptide monomers in solution, the purified product of reactions (white powder) was dissolved in several solvents (water, methanol, and buffers at pH 6, 8, and 10). At pH 6, the samples acquired a crystalline aspect, whereas at alkaline pH, oligo(Phe) showed better solubility. No significant differences in fiber morphology between reactions at different concentrations of enzyme were observed, suggesting that the enzyme did not affect the self-assembling behavior. Purified oligo(Phe) self-assembled into fibers in aqueous solution, and after heating 10 mg/mL at 85 °C, oligo(Phe) formed a gel composed of long, 1-μm-diameter fibers (Figure S4). These fibers can be also observed after drying oligo(Phe) in 60% methanol. Self-assembly was more evident in

samples with a higher degree of hydrolysis, as with L-Phe-Met or in reactions carried out in carbonate buffer, which can be explained by the presence of ethyl ends in oligo(Phe) that can reduce the ionic interactions that occur more favorably with carboxylic ends in fiber formation.[17] Samples with ethyl ends tend to form long crystal structures. Moreover, structural configuration of the fibers can also affect proteolysis: Ryu *et al*.[28] reported that the susceptibility of Phe-Phe nanotubes to proteolytic attack by pro K changed with their morphology, with nanowires being more resistant to proteolysis than nanotubes. Our results indicate that oligo(Phe) fibers are not hollow, being more akin to flat wires than to tubes, and show morphologies that appear in amyloid peptides[29] or pentafluorophenylalanine[18] fibers. Because the flat morphology can help oligo(Phe) resist hydrolysis, the conformation of the peptide could affect the degree of hydrolysis as well as the length of the product.

The TREN(Phe) synthesis reaction mixtures showed complex crystals and fibers depending of the TREN:monomer ratio used, resembling oligo(Phe) more in samples with ratios 1:15 and 1:30. Purified samples, which were yellowish white to white powders, formed fibrils water evaporated. Under polarized light, the fibers appeared discontinuous, suggesting a twisted fibril structure (Figure 5A), which was more evident in samples with lower TREN:monomer ratios (1:6 and 1:9). Unlike oligo(Phe) that remained soluble, TREN(Phe), in the 1:6 ratio samples, self-assembled into fibers in 60% methanol after 1 h at 24 °C at 10 mg/mL. Laser scanning microscopy showed that TREN(Phe) fibers auto-fluoresced at 405 nm (Figure 5B), with the fluorescence intensity being proportional to TREN concentrations (data not shown). The observation of fluorescence confirmed the conjugate synthesis of TREN and oligo(Phe).

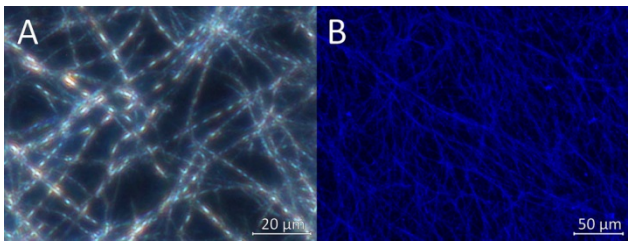


Figure 5. Microscopic observation of dry TREN(Phe) samples. **A**. Optical micrograph under polarized light. **B**. Fluorescence micrograph, with excitation at 405 nm.

Phenylalanine peptides can form multiples structures depending on their concentrations or reaction conditions used.[16, 28] To simplify comparisons of fiber formed by peptides with different branched structures, only low and high TREN:monomer ratios (1:30 and 1:6) were included. When samples were dissolved in 60% methanol and allowed to dry immediately, self-assembly was observed after the solvent evaporated (Figure 6A-C). Oligo(Phe) samples showed radial growth of straight fibers, 1:30 TREN(Phe) showed branched fibers, and 1:6 TREN(Phe) showed branch structures and spheres (as reported for chemically synthetized $(Phe\text{-}Phe)_3$TREN conjugates[19]). SEM imaging of oligo(Phe) revealed 1-μm-diameter fibers attached to mica, whereas the TREN-based peptides presented fibers 50 nm in diameter that matted into thicker fibers that were 150 nm and 1 μm in diameter (Figure 6D-F). The 50-nm-diameter fibers were similar in size to Phe-Phe nanotubes described previously[17, 24, 28, 30] and to other LMWGs,[18, 31, 32] but their non-hollow, flat structure was unlike the structure of dipeptide nanotubes. Fibers of TREN(Phe) peptides were interconnected and formed complex scaffolds. In agreement with observations using optical microscopy (Figure 6A-C), SEM revealed that TREN enhanced flexibility in the interaction between oligo(Phe) peptides by increasing fiber connections (Figure 6D-F). Furthermore, peptides with properties similar to Phe-Phe or longer oligomer could be

obtained by controlling branching-aspect ratios, which, in turn, can be controlled by the monomer:TREN ratio in reactions.

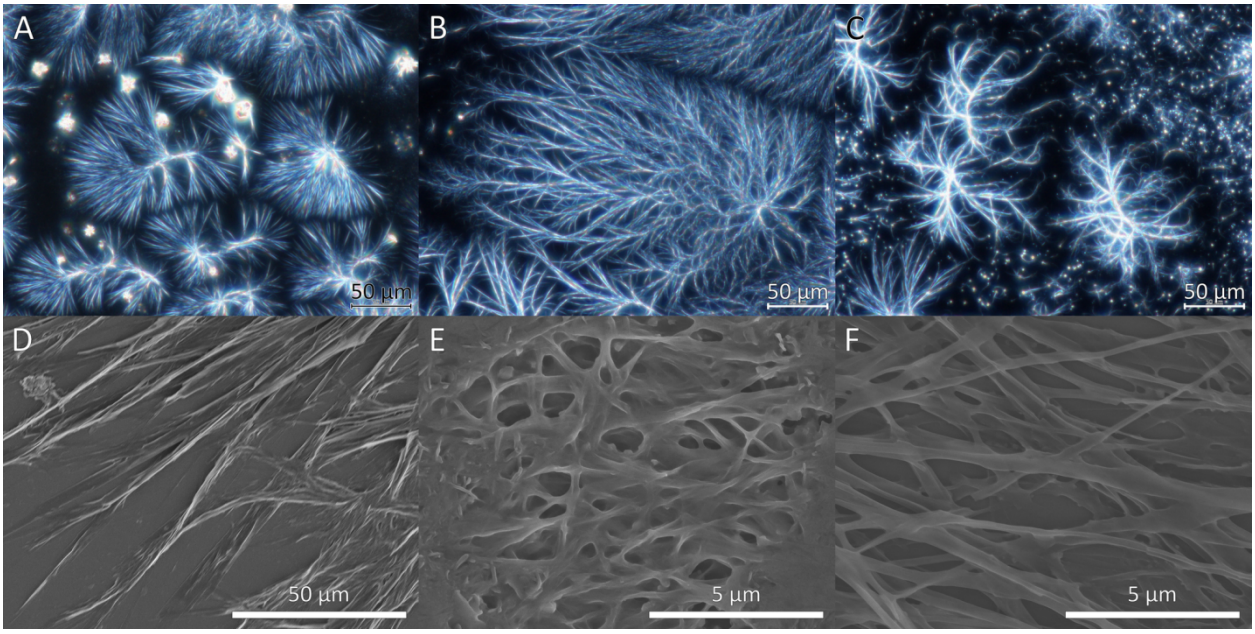


Figure 6. A-C. Dark field microscopy of dry film fibers. A. Oligo(Phe). B. 1:30 TREN(Phe). C. 1:6 TREN(Phe). D-F. Scan-ning electron micrographs of 60% methanol xerogels. D. Oligo(Phe). E. 1:30 TREN(Phe). F. 1:6 TREN(Phe).

Cytotoxicity assays. Because oligo(Phe) and TREN(Phe) formed fibrous structures resembling amyloid fibers, their potential toxicity was evaluated by incubating Human Embryonic Kidney 293 (HEK 293) cells for 8 h with several concentrations of oligo(Phe), TREN(Phe), and l-phenylalanine (PheOH). Control experiments were conducted using the same volume of Dulbecco's phosphate-buffered saline. PheOH has been reported to be cytotoxic at high concentrations.[33] The peptides assayed here showed no more toxicity than PheOH. When oligo(Phe) was compared with amyloid peptides (cytotoxic peptide), the cytotoxicity of

oligo(Phe) was nearly 300-times lower than that of amyloid peptides,[34] and 36-times lower than that of the hydrolyzed products of amyloid peptides.[35]

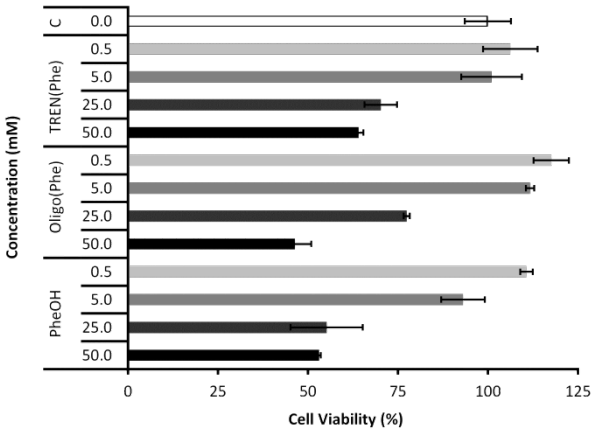


Figure 7. HEK 293 cell viability after 8 h in the presence of several concentrations of l-phenylalanine (PheOH), oligo-phenylalanine [oligo(Phe)], oligo-phenylalanine:TREN [TREN(Phe)], and negative control (C). Error bars represents the standard deviation of replicates

These results demonstrate that phenylalanine-based peptides can be used for at least 8 h in biological environments without producing high toxicity. The low cytotoxicity observed with TREN(Phe) peptides is similar that reported for polyphenylalanine-grafted hyperbranched polyethylenimine in gene transfection,[36] which opens up possibilities for new applications of these polymers. Oligo(Phe) and TREN(Phe) peptides show similar cytotoxicity levels as silk β-sheet peptides, which also form fibril scaffolds.[37] Collectively, the above findings indicate that bioconjugates between oligo(Phe) and TREN are not significantly cytotoxic and that they are sufficiently biocompatible to be used in biomedical applications, for example in the development of tissue-engineering scaffolds.

Conclusions

This work evaluated the suitability of pro K as an enzyme for KCS of hydrophobic peptides. Pro K is one of the most widely used proteases because of its high proteolytic activity, but the high processivity of pro K can be converted to synthesis by using a high substrate/enzyme ratio. Given the affinity of pro K for aromatic amino acids, the synthesis of phenylalanine oligomers by pro K was examined and the effects of various conditions on yield and product length were measured. Polymer length could be controlled by using distinct buffers (DP_{avg} 4.7 to 12), and the hydrolysis of ester ends could be promoted by substrate engineering (changing ethyl to methyl ester increased hydrolysis by 70%) or by modifying pH and temperature. The product obtained formed amyloid-like fibers that self-assembled into a fibrous network depending on concentration, pH, and temperature; self-assembly was promoted by unprotected carboxylic ends. Conversely, we synthetized branched TREN polymers by KCS and were able to influence the average length and yield of the polymer by modifying the terminator:monomer ratio. TREN(Phe) could self-assemble into fluorescent fibers in 60% methanol or organic solvents. These star peptides showed the propensity to develop interconnections robustly during fibril formation. Despite forming amyloid-like fibers, the phenylalanine oligomers were not found to be more cytotoxic than their hydrolysis product phenylalanine. Pro K is a suitable enzyme for peptide synthesis because it responds to reaction modifications, which makes it possible to engineer the reaction process to generate diverse products. The high cost of pro K compared with that of other proteases is compensated for by the low amounts of pro K required for reactions and its high enzymatic activity. The combination of high affinity for certain amino acids and low overall specificity makes pro K an ideal candidate for further developing precise peptide synthesis methods.

ASSOCIATED CONTENT

Supporting Information.

Figure S1. 2D NMR study of oligo(Phe); **Figure S2**. 2D NMR study of TREN(Phe) 1:6; **Figure S3**. Fourier transform infrared spectra of oligo(Phe) and TREN(Phe); **Figure S4**: Self-assembling pattern of oligo(Phe). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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