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Influence of N_ε-Protecting Groups on the Protease-Catalyzed Oligomerization of L-Lysine Methyl Ester

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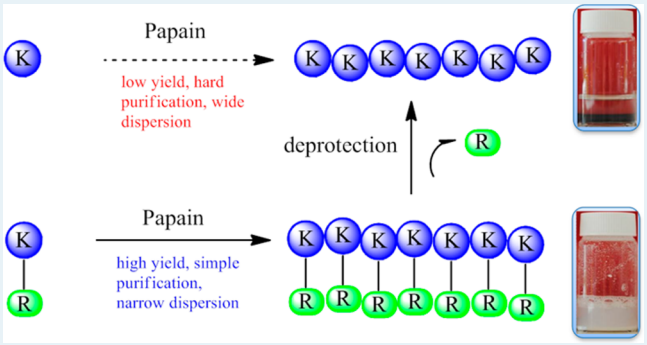
Supporting Information

ABSTRACT: The direct oligomerization of L-lys-OMe by bromelain catalysis gave oligo(L-lys) with DP_{avg} ~ 3.6 and dispersity ~ 1.1. Since higher chain length oligo(L-lys) with lower dispersity values and one reactive amine for selective conjugation would be beneficial, we explored protease-catalyzed oligomerization of N_ε-protected L-lys monomers where N_ε-groups included *tert*-butoxycarbonyl (Boc) or benzyloxycarbonyl (Z) groups. By using N_ε-protected L-lys monomers, oligopeptide side-chains are hydrophobic-neutral which should dramatically alter enzyme kinetic and binding constants relative to nonprotected L-lys. Schechter and Berger's conceptual model guided our choice of papain as the protease catalyst. Papain-catalyzed oligomerization of N_ε-Boc-L-Lys-OMe gave products with DP_{avg} values that were pH dependent and varied from 4.7 ± 0.2 to 7.5 ± 0.1. Similarly, oligo(N_ε-Z-L-Lys) synthesis was pH dependent, and DP_{avg} values varied from 4.3 ± 0.2 to 5 ± 0.2. Oligo(N_ε-Boc/Z-L-Lys) that precipitates from reaction media had a low dispersity (~1.01). The relatively smaller N_ε-Boc group should increase propagating chain solubility enabling oligopeptides to reach higher DP_{avg} values prior to their precipitation. Since papain-catalyzed oligomerizations of N_ε-Boc/Z-L-Lys proceeded slowly at 0.54 mg/mL, higher enzyme concentrations were studied. By increasing the enzyme concentration in oligomerizations from 0.54 to 1.62 mg/mL for 3 h reactions, the %-yield and DP_{avg} of oligo(N_ε-Z-L-lys) increased from 24 ± 0 to 88 ± 2 and 4.1 ± 0.7 to 5.7 ± 0.1, respectively. Furthermore, at 1.89 mg/mL papain, the %-yield of oligo(N_ε-Z-L-lys) increased with time reaching 91% in 2 h. Acetonitrile at 20%-by-volume was a useful cosolvent that increased the oligopeptide yield and DP_{avg} relative to reactions run in pure buffer.



KEYWORDS: *protease-catalysis, oligopeptide, oligolysine, N_ε-Z-L-lysine, N_ε-Boc-L-Lys*

INTRODUCTION

Oligo(L-Lys) alone—conjugated with a biological molecules such as lipids, oligosaccharides, and hydrophobic amino acid oligomers—and as pendant groups on synthetic polymer chains have shown a wide range of promising properties. Examples of applications in which the unique characteristics of oligo(L-Lys) have been explored are the following: (*i*) interaction with DNA for potential use in gene delivery,[1] (*ii*) self-assembling amphiphilic nanostructures that form hydrogel scaffolds;[2] (*iii*) acid sensitive micelles for drug delivery,[3] (*iv*) complexation of negatively charged therapeutics such as insulin and heperin,[4] (*v*) formation of nanoscale vesicles and double emulsions,[5] (*vi*) formation of complexes with metals such as iridium(III) for anticancer drug delivery;[6] (*vii*) as components in antimicrobial molecules and materials,[7] and (*viii*) enhancing the efficiency of photo sensitizers for photodynamic therapy.[8] Furthermore, the properties of oligolysine in many of the above applications is highly dependent on oligo(L-Lys) chain length where it is generally preferable to work with oligo(L-Lys) with chain lengths greater than 3. Examples include enhancing peptide-microgel interactions as the oligo(L-Lys) chain length is increased from 3 to 5 and 10,[9] mineralization of titanium dioxide where oligo(L-Lys) with chain lengths of more than three lysine units is needed to induce fast precipitation,[10] and *N*-(2-hydroxypropyl)methacrylamide (HPMA)-*g*-oligo(L-Lys) transfected cultured cells with significantly higher efficiencies when the oligo(L-Lys) chain length was 5 and 10 units.[1b] The need to increase the chain length of oligo(L-Lys) synthesized by protease catalysis was an important motivating factor for the research described in this paper.

The synthesis of oligo(L-Lys) has relied on solid phase peptide synthesis (SPPS), liquid phase peptide synthesis (LPPS), and ring-opening polymerization (ROP) of α-amino acid N-carboxylic anhydrides (NCAs). SPPS and LPPS provide peptides with uniform chain lengths and sequences. However, the synthesis is inefficient due to repetitive protection/deprotection reactions in which one amino acid at a time is added to elongate the chain.[11] Furthermore, toxic reagents and

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large amounts of organic solvents are required. Consequently, the resulting products are expensive, which limits their use to primarily therapeutic and specialty cosmetic applications.[12] The alternative route to peptides is via N-carboxy anhydride (NCA) monomer synthesis and ROP. However, this process requires toxic phosgene or phosgene equivalents, strict water removal, and high monomer purity.

The above supports the need to develop alternative peptide synthetic methods which are safe, scalable, and cost-effective. Protease-catalyzed peptide synthesis provides such an alternative since it circumvents the aforementioned difficulties associated with conventional synthetic approaches discussed above.[13] While proteases function in nature to catalyze peptide bond hydrolysis reactions, by proper manipulation of the reactions physiological conditions, proteases can also catalyze peptide bond-forming reactions with surprising efficiency.[13d,e,14] In a kinetically controlled protease-catalyzed synthesis, the active site serine and cysteine hydroxyl and thiol functionalities, respectively, undergo a nucleophilic attack on the substrate carbonyl carbon (normally an amino acid ester) to form an acyl-enzyme intermediate [E·S] with ethanol liberation. Aminolysis then occurs by deacylation of the acyl-enzyme intermediate resulting from nucleophilic attack by the α-amino group of an amino acid or oligomer to form a peptide bond.[13d,14a,15]

Given the importance of oligo(lysine) (see discussion above), it is surprising that the literature has been relatively silent on its synthesis via protease catalysis. An important exception is the pioneering work by Aso and Kodaka in 1992 who first described results on a protease-catalyzed synthesis of oligo(L-lys).[16] They used trypsin as the catalyst for L-lys-Et oligomerization in a sodium carbonate buffer. Analysis by HPLC using online ninhydrin derivatization gave values of free amino acid remaining in products. Studies were conducted to determine how the medium pH, reaction time, and other parameters influence oligo(L-lys) yield and initial reaction rate (V_{app}). They reported an overall reaction yield above 70% after 2 h at pH 10. While Aso and Kodaka[16] provided this important first example that oligo(L-lys) can indeed be prepared by protease-catalysis, they did not quantify DP_{avg}, chain length distribution, and free L-lys formation (hydrolysis instead of amidation reactions). Such data are needed to understand the relative importance of competing reactions during oligomer formation. Inspired by Aso and Kodaka,[16] our group extended their work by exploring additional proteases for L-lys-Et oligomerization.[17] Furthermore, NMR and HPLC-UV-MS methods were developed that allowed determination of %-monomer conversion, average oligo(L-lys) chain length (DP_{avg}), and the distribution of chain lengths in product mixtures.[17,18] A mechanism was presented that describes the sequence of reactions believed to occur during conversion of L-Lys-Et to oligo(L-Lys).[17] Papain, bromelain, α-chymotrypsin, and trypsin were evaluated to determine their relative activities for conversion of L-Lys-Et to oligo(L-Lys). The highest percent conversion values were obtained by bromelain and trypsin catalysis (76 ± 3 and 68 ± 1%, respectively) at pH values of 7 and 10, respectively. Overall, bromelain gave the highest value of both DP_{avg} (4.1) and percent monomer conversion (84 ± 2%). Analysis of products formed by bromelain catalysis (pH 7.6, 40 °C, 20 mg/mL bromelain, and 0.5 M L-Lys-Et) as a function of reaction time showed that oligomers with chain lengths longer than 10 are formed within 5 min. Furthermore, at 30 min, the DP_{avg} and longest oligomer chain lengths reached maximum values of ∼3.6 and 12.0, respectively.

Previous work has shown that side chain protected amino acids can be effective acyl donors and acceptors for dipeptide synthesis. For example, Gill and co-workers reported that, by using α-chymotrypsin as a catalyst and N_ε–Z–L-Lys(Z)-OEt (Z is a benzyloxycarbonyl group) as the acyl donor, N_ε–Z–L-Lys-Gly-OEt was prepared in yields approaching 82%.[19] Furthermore, Jakubke and Beck-Piotraschke[20] reported that, by using trypsin as catalyst, N_ε–Z–L-Lys-NH_2 as the acyl acceptor and a histidine ester as the acyl donor, dipeptides were prepared in 80% yield.[20] However, the synthesis of longer oligomers with repetitive N_ε–Z–L-Lys units has not been reported. A protease catalyzing the synthesis of such a peptide would need to have multiple adjacent subsites that could accommodate repeat units bearing large hydrophobic amino acid side chains.

During the course of our research on L-lys-Et oligomerization,[17] the following challenges were encountered: (*i*) the dual aqueous solubility of the enzyme, unreacted monomer, and product that requires a separation step, (*ii*) a broad distribution of oligo(L-lys) chains ($Đ_M$ is about 1.1) and (*iii*) low DP_{avg} values (up to about 4) relative to previous work where the product precipitated after reaching a limited range of chain lengths dictated by product solubility,[21] (*iv*) the need to quench reactions so that the desired oligo(L-lys) kinetic product is obtained, and (*v*) free amino acids in side chains do not allow for selective end-group conjugation to various molecules (e.g., lipids, monomers, polymers, and nanoparticles). For *i*, separation of the protein was achieved by ultrafiltration using a 3 K_d filter, but oligo(L-lys) and residual monomer remain as a mixture. With regard to *iii*, oligomerization reactions of hydrophobic amino acid ethyl esters such as α,γ-diethyl-glutamate[21a] and phenylalanine ethyl ester[21b] and alanine-glycine alternating oligopeptides[22] did not require careful attention to reaction time since the oligo(peptide) is separated from the protein by its precipitation from the reaction media that also thermodynamically drives the reaction toward product formation. Furthermore, precipitation of oligomers upon formation will decrease the tendency for competing reactions such as transamidation and hydrolysis to occur as the monomer concentration decreases. These competing reactions can lower DP_{avg} and increase the product chain length distribution.[20]

For the above reasons, this paper explores protease-catalyzed oligomerization of L-lys monomers protected at the N_ε-position with either *tert*-butoxycarbonyl (Boc) or benzyloxycarbonyl (Z) R-groups (see route 2, Scheme 1). Boc- and Z- were selected since (*i*) they are two of the most widely used protecting groups in chemical peptide synthesis and (*ii*) they have different sizes that allow us to probe enzyme–substrate specificity.[23] Furthermore, their protection–deprotection chemistry has been well studied, and both protection groups can be easily removed after oligomerizations. Papain was selected as the protease based on a hypothesis described in detail below which suggested it would function well with amino acid monomers bearing large hydrophobic groups. Analysis of DP_{avg} and the distribution of oligo(N_ε-Z-L-Lys) and oligo(N_ε-Boc-L-Lys) chains in precipitated products were performed by ^{1}H NMR spectroscopy, MALDI-TOF, and HPLC-UV-MS (abbreviations defined in the Experimental Section).

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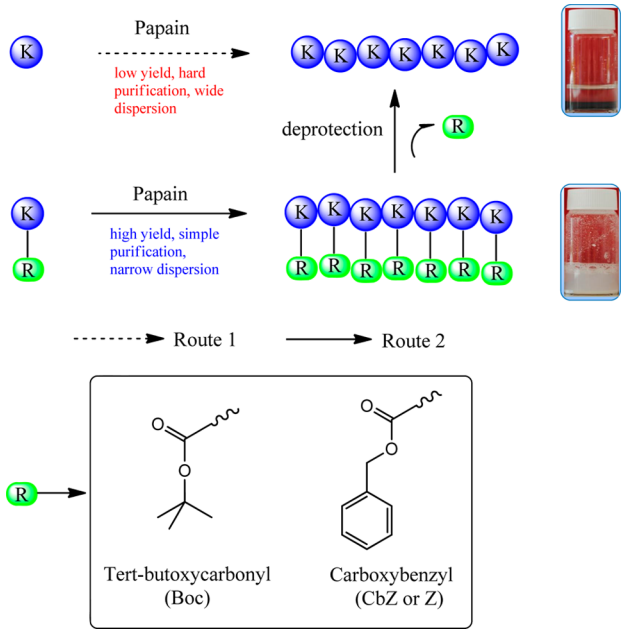
Scheme 1. Synthesis of Oligo(L-Lys) via Direct Oligomerization of L-Lys-OMe (Route 1) or Using the N_ε-Protected Monomer L-Lys[R]-OMe (Route 2)[a]



[a]For the latter, R groups studied include *tert*-butoxycarbonyl (Boc) and benzyloxycarbonyl (CbZ or Z).

EXPERIMENTAL SECTION

Materials. L-lys-Et·HCl_2, L-lys-Me·HCl_2, N_ε-Boc-L-Lys-OMe·HCl, N_ε-Z-L-Lys-OMe·HCl, trifluoroacetic acid (TFA), methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), hydrogen bromide solution (33 wt % in acetic acid), hexafluoro-2-propanol (HFIP), deuterated trifluoroacetic acid (CF_3COOD), and α-cyano hydroxycinnamic acid (CHCA, MALDI-TOF matrix) were all purchased from Sigma-Aldrich and used directly as received. Crude papain (EC # 3.4.22.2; source-*Carica papaya*; 30 000 USP units/mg of solid; molecular weight 21K) was purchased from CalBioChem. Water-insoluble materials in the as-received papain were removed by a literature protocol.[21a] Deionized water (DI, 18.2 MΩ·cm purity) was obtained from a RIOS 16/MILLQ synthesis Millipore water purification system.

Enzyme. Crude papain (EC # 3.4.22.2; source-Carica papaya; 30 000 USP units/mg of solid; molecular weight 21K) was purchased from CalBioChem. The enzyme concentrations reported are based on determination of papain content within the crude extract. First, the protein content of the crude extract was determined using the bicinchoninic acid (BCA) protein assay. Subsequently, the content of papain relative to other proteins in the mixture was determined using SDS PAGE analysis. From this analysis the content of papain in the crude extract was found to be 2.7%-by-wt (Supporting Information).

Synthetic Methods. *General Procedure Followed for Protease Catalyzed Oligo(L-lys) Synthesis.* The method used is identical to that previously published with the following modifications.[14] L-lys-OMe·HCl (466.28 mg, 2 mmol), 2 mL of water, and 2 mL (0.9 M) of phosphate buffer solution were transferred to a 50 mL Falcon tube. A Tiamo automatic titration system and a Metrohm CH9101 dosing unit were used to control the pH as was previously described.[17] The flask was gently stirred in a water bath at 40 °C for a predetermined reaction time while the pH value was held constant by the pH stat. The protease was then removed from the mixture by centrifugation using an Amicon Ultra-15 centrifugal filter with a 3000 molecular weight cutoff membrane. The resulting product was lyophilized for 2 days to give a white powder solid.

General Procedure Followed for Protease Catalyzed Oligo(N_ε-Boc-L-Lys) Synthesis. N_ε-Boc-L-Lys-OMe·HCl (593.6 mg, 2 mmol), 2 mL of water, and 2 mL of phosphate buffer solution (0.9M) were transferred to a 50 mL Falcon tube. The falcon tube was gently stirred in a water bath at 40 °C for a predetermined reaction time while the pH value was held constant by the pH stat. The reaction mixture was then centrifuged at 8000 rpm. The precipitate was washed three times with a pH = 5 diluted HCl solution before lyophilizing for 2 days to give a white powder solid from which the %-yield of oligomeric product was calculated.

General Procedure Followed for Protease Catalyzed Oligo(N_ε-Z-L-Lys) Synthesis. N_ε-Z-L-Lys-OMe·HCl (661.6 mg, 2 mmol), 2 mL of water, and 2 mL of phosphate buffer solution (0.9M) were transferred to a 50 mL Falcon tube. The falcon tube was gently stirred in a water bath at 40 °C for a predetermined reaction time while the pH value was held constant by the pH stat. The reaction mixture was then centrifuged at 8000 rpm. The precipitate was washed with a pH = 2 diluted HCl solution three times before lyophilizing for 2 days to give a white powder solid from which the %-yield of oligomeric product was calculated.

Papain-Catalyzed Oligo(N_ε-Z-L-Lys): Determination of %-yield and DP_{avg} Values As a Function of Reaction Time. Separate N_ε-Z-L-Lys-OMe·HCl (661.6 mg, 2 mmol) oligomerization reactions were conducted using papain (0.54 and 1.89 mg/mL) as a catalyst, in 4 mL of phosphate buffer solution (0.45 M), at pH 7, for predetermined reaction times. Determination of DP_{avg} was by ^{1}H NMR.[17]

General Procedure Followed to Deprotect Oligo(N_ε-Boc-L-Lys). Oligo(N_ε-Boc-L-Lys) (1 g) was suspended in 5 mL of a 2 N HCl solution and gently stirred overnight. The clear solution obtained was lyophilized for 2 days to give a solid.

General Procedure Followed to Deprotect Oligo(N_ε-Z-L-Lys). Oligo(N_ε-Z-L-Lys) (500 mg) and 10 mL of HBr/AcOH solution (HBr 33 wt %) were transferred to a 50 mL round-bottom flask and gently stirred for 5 h. The solution was then added drop-by-drop to 200 mL of diethyl ether until a precipitate formed. The precipitate was filtered and washed three times in 30 mL of diethyl ether. The washed precipitate was then dried in a vacuum oven to give a white powder solid.

Instrumental Methods. *Nuclear Magnetic Resonance (NMR) Spectroscopy.* Proton (^{1}H) NMR spectra were recorded on a Bruker DPX 300 spectrometer at 300 MHz. NMR experiments of oligo(N_ε-Boc-L-Lys) and oligo(N_ε-Z-L-Lys) were performed at 10 mg/mL with a data acquisition delay of 1 s and a total of 128 scans in DMSO-d_6 and DMSO-d_6/TFA-d cosolvent (volume 30:1), respectively. Data were collected and analyzed by MestRe-C software. Proton chemical shifts were referenced to tetramethylsilane (TMS) at 0.00 pm.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF). MALDI-TOF spectra were obtained on an OmniFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Inc.). The instrument was operated in a positive ion reflectron mode with an accelerating potential of +20 kV. The TOF mass analyzer had pulsed ion extraction. Omni-FLEX

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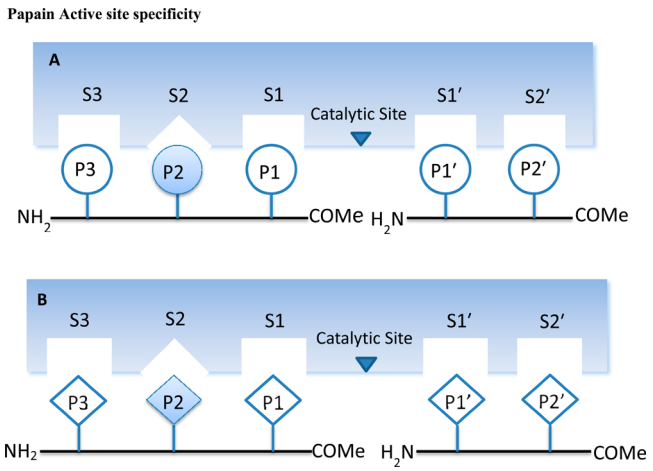
TOF control software was used for both hardware control and calibration while an X-mass OminFLEX 6.0.0 was used for data processing. The spectra were acquired by averaging at least 400 laser shots. The pulsed ion extraction delay time was 200 ns. To formulate the matrix solution, a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) was prepared in trifluoroacetic acid/acetonitrile/H_2O (TA) 0.1-to-33-to-100 v/v. Oligopeptide samples were dissolved in hexafluoroisopropanol (HFIP), diluted to 1−5 pmol/μL with TA solution, and then mixed with an equal volume of a saturated matrix solution. This mixture (1 μL) was then applied onto the clean target which was subsequently dried in a stream of cold air. The relative intensity threshold was set so that the peaks with intensity values ≤1% of the highest peak were considered as noise and removed from the database.

HPLC-UV-MS Analysis. The HPLC_UV-MS system was composed of a Waters Alliance 2795 Separation Module (Milford, MA, USA) coupled with a Waters 2996 photodiode array detector and a Waters ZQ detector with an electrospray ionization probe. Analyte (3.5 mg/mL) was injected onto a Waters XBridge Shield RP18 column (50 mm-4.6 mm i.d.; pore size 135 Å, particle size 3.5 μm) that was kept in a 35-C column oven.[17,24]

RESULTS AND DISCUSSION

Papain Active Site Specificity. Various laboratories including ours have demonstrated that papain is highly efficient in catalyzing peptide synthesis.[17,21a,f,g,26] Previously, our team reported on the relative activities of papain, bromelain, α-chymotrypsin, and trypsin for L-Lys-OEt oligomerization.[17] Papain was less efficient than bromelain and α-chymotrypsin based on both the %-yield and the DP_{avg} of synthesized oligo(L-lys).[17] Herein, we explored the potential use of papain as a catalyst for oligomerizations of N_ε-protected L-lys-Boc-Me and L-lys-Z-Me. Schechter and Berger used a conceptual model to describe the specificity of a protease where each subsite accommodates the side chain of a single amino acid residue.[25] According to this system, the protease active site is envisioned as having a series of subsites (S) which interact with the amino acid units (P) of the peptide or protein substrate (see Scheme 2).[27] The arrow indicates the catalytic site of the protease between residues P_1−P_1', where bond hydrolysis occurs. Based on this nomenclature, the specificity of numerous proteases has been documented for both their hydrolytic and synthetic properties. The kinetic and structural data proposed by Turk et al.[28] for papain assign five subsites (S1, S2, S3, S1′, S2′), shown in Scheme 2, that are important for substrate binding. Scheme 2 was adapted so that circular P-sites represent nonprotected L-lys-moieties (Scheme 2A) whereas N_ε-protected by Boc- or Z-groups are depicted as diamonds (Scheme 2B). Square shaped papain subsites (S) are highly promiscuous as they can accommodate any amino acid side-chain, whereas triangular shaped subsites are selective for hydrophobic amino acid units. The fact that papain possesses a preponderance of square shaped S-sites indicates it has a broad specificity. This enables papain to cleave peptide bonds found along a wide-range of peptide or protein sequences. However, papain does have a preference for bulky hydrophobic amino acid units (e.g., Phe, Leu, Ile) at its S2 site (triangular). Consequently, bond formation between L-lys-OMe units should progress to form dimers or trimers while further chain growth would be hampered due to unfavorable interactions at the S2 site that leads to poor binding of propagating oligo(L-lys). Indeed, this

Scheme 2. Mechanism of Protease-Catalyzed Oligomerization of (A) L-Lys-OMe and (B) N_ε-Z-L-lys-OMe (Z = Boc- or Z-)[a]



[a]Nomenclature of S- and S′-subsites in the protease active site and substrate P- and P′-residues according to Schechter and Berger.[25]

model is consistent with experimental results in which %-monomer conversion and DP_{avg} of papain-catalyzed L-lys-OEt oligomerization did not exceed about 20% and 3.0, respectively. However, based on the above model, we hypothesized that by changing the monomer from L-lys-OMe to N_ε-protected L-lys-OMe with Boc and Z-groups, favorable binding of papain subsites with L-lys[Boc] and L-lys[Z] units along peptides should result allowing formation of oligo(N_ε-Boc-L-lys) and oligo(N_ε-Z-L-lys) with DP_{avg} values substantially higher than 3. The work discussed below tests this hypothesis. Furthermore, we wish to exploit that if oligo(N_ε-Boc-L-lys) and oligo(N_ε-Z-L-lys) reach suitable chain lengths, the corresponding products will be insoluble separating from the aqueous phase where the protein resides. This will shift the equilibrium to product formation and decrease the propensity for unfavorable reactions such as transamidation.

Structural Analysis. *HPLC Analysis.* Figure 1a and b display the UV (220 nm) chromatograms of oligo(L-Lys)s deprotected from oligo(N_ε-Boc-L-lys) and oligo(N_ε-Z-L-lys), respectively. The molecular weight of each peak was confirmed by MS detection in full scan mode (spectrum not shown). Inspection of the UV chromatograms in Figure 1a and b shows that the analyte is a mixture of oligo(L-Lys) chains consisting of components with lengths from 5 to 8 units and from 4 to 6 units, respectively. The majority of product peaks correspond to oligomers that are de-esterified. This is expected since the methods of N_ε-deprotection will also remove methyl ester groups at oligomer C-terminal positions. The DP_{avg}, M_n, and the dispersity ($Đ_M$) of deprotected oligo(L-Lys) were calculated following a previously published method[17,24,29] from relative peak areas of constituent oligo(L-Lys) molecules that differ only in chain length. When the UV trace was used, we assumed that the peak area of each component in the mixture directly reflected its respective concentration. In contrast, the calculation of DP_{avg} from the UV chromatogram took into account that the peak areas are influenced by both the number of peptide bonds and the concentration of each component.[30] Since the UV absorbance of a constituent oligo(L-Lys) species is directly proportional to the number of peptide bonds in the molecule,[29] and each $(Lys)_i$ oligomer contains $i - 1$ peptide

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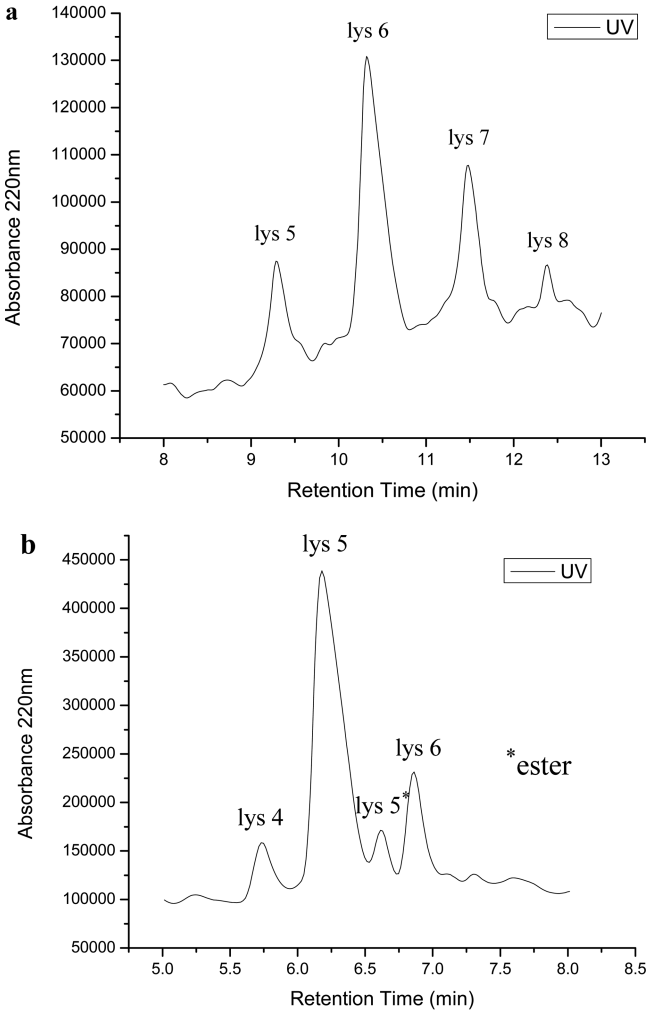

Figure 1. HPLC-UV chromatograms of oligo(L-Lys) after deprotection from (a) oligo(N_ε-Boc-L-lys) and (b) oligo(N_ε-Z-L-lys). Oligomerizations were conducted by papain catalysis (1.62 mg/mL) with 0.5 M monomer concentration at pH 7 for 3 h.

bonds, the DP_{avg}, M_n, and $Đ_M$ of oligo(L-Lys) were determined by the following relationship using experimentally determined peak integration values from HPLC-UV:

$$DP_{avg} = \frac{\sum_2^i \left[i * \frac{UV_i}{(i-1)} \right]}{\sum_2^i \left(\frac{UV_i}{(i-1)} \right)}$$

$$M_n = \frac{\sum_2^i \left[M_i * \frac{UV_i}{(i-1)} \right]}{\sum_2^i \left(\frac{UV_i}{(i-1)} \right)}$$

$$M_w = \frac{\sum_2^i \left[M_i^2 * \frac{UV_i}{(i-1)} \right]}{\sum_2^i \left(M_i * \frac{UV_i}{(i-1)} \right)}$$

$$Đ_M = \frac{M_w}{M_n}$$

where "i" is the number of lys units in $(Lys)_i$ of oligo(L-Lys) ($i \geq 2$), UV_i is the UV absorbance of $(Lys)_i$ in the HPLC-UV spectrum and M_i is the molecular weight of $(Lys)_i$. As one example, since the peaks corresponding to lys_5, lys_6, lys_7, and lys_8 were observed in the HPLC-UV chromatogram of oligo(L-Lys) shown in Figure 1a, the DP_{avg} of the product is determined by solving the following equation:

$$DP_{avg} = \frac{5*\frac{UV_5}{4} + 6*\frac{UV_6}{5} + 7*\frac{UV_7}{6} + 8*\frac{UV_8}{7}}{\frac{UV_5}{4} + \frac{UV_6}{5} + \frac{UV_7}{6} + \frac{UV_8}{7}}$$

Using the above equations, the DP_{avg}, M_n, and $Đ_M$ of oligo(L-Lys)'s shown in Figure 1a and b are calculated by equations above are 6 and 4.9, 794 and 645, and 1.01 and 1.02, respectively. Percent yield is determined gravimetrically based on the weight of precipitated product relative to that of the initial monomer in the feed corrected for losses of methanol during amide bond formation. Product DP_{avg} was also determined by integration of ^{1}H NMR peaks as described below based on a previously published method.[17]

^{1}H NMR Analysis. ^{1}H NMR spectra of oligo(N_ε-Boc-L-lys) and oligo(N_ε-Z-L-lys) are displayed in Figure 2A and B, respectively. In Figure 2A, assignments of peaks A, B, C, D, E, E′, E″, L, and M are based on comparisons to the ^{1}H NMR spectrum of N_ε-Boc-L-lys-ome (Figure S1). The methine protons F, G, and H were assigned based on comparison to literature assignments of oligo(L-lys).[17] Since the number of N- or C-terminal N_ε-Boc-L-lys units is equal to the number of oligo(N_ε-Boc-L-lys) chains, DP_{avg} values of oligo(N_ε-Boc-L-lys) were determined by the following relationship using experimentally determined peak integration values:

$$DP_{avg} = (F + G + H)/F$$

Similarly, in Figure 2B, the assignment of peaks A, B, C, D, E, F, G, H, H′, H″, O, and P are based on comparisons to the ^{1}H NMR spectrum of N_ε-Z-L-lys-OMe (Figure S2) The methine protons I, J, and K were assigned based on comparison to literature assignments of oligo(L-lys).[17] The DP_{avg} values of oligo(N_ε-Z-L-lys) was determined by the following relationship using experimentally determined peak integration values:

$$DP_{avg} = (I + J + K)/I$$

Comparison of Papain-Catalyzed Oligomerizations of L-lys-OMe, N_ε-Boc-L-lys-OMe, and N_ε-Z-L-lys-OMe. Table 1 compares the %-yield, DP_{avg}, M_n, and $Đ_M$ of oligo(L-Lys) synthesized by papain-catalyzed (0.54 mg/mL) (1) direct oligomerization of L-Lys-OMe, (2) oligo(N_ε-Boc-L-lys) after deprotection of the N_ε-Boc groups, and (3) oligo(N_ε-Z-L-lys) after deprotection of the N_ε-Z groups. By carrying out oligomerizations on N_ε-Boc and N_ε-Z-protected L-Lys-OMe monomers instead of by direct oligomerization of L-Lys-OMe, a large increase in the DP_{avg} was observed while $Đ_M$ decreases. Hence, oligomers of L-Lys units with higher chain length averages and lower dispersity were prepared by using N_ε-protected L-Lys-OMe monomers that precipitate from solution after reaching DP_{avg} values >4. Table 1 also shows that values of DP_{avg} determined by HPLC-UV and ^{1}H NMR[17] are in close agreement.

Effect of Reaction pH on Papain-Catalyzed Synthesis of Oligo(N_ε-Boc-L-Lys) and Oligo(N_ε-Z-L-Lys). Previous studies on protease-catalysis demonstrated that the optimal pH to hydrolyze and synthesize peptide bonds can differ substantially.[31] Furthermore, protease activity for oligopeptide synthesis is pH-dependent.[17,21a,b,d−f,32] The pH dependence for

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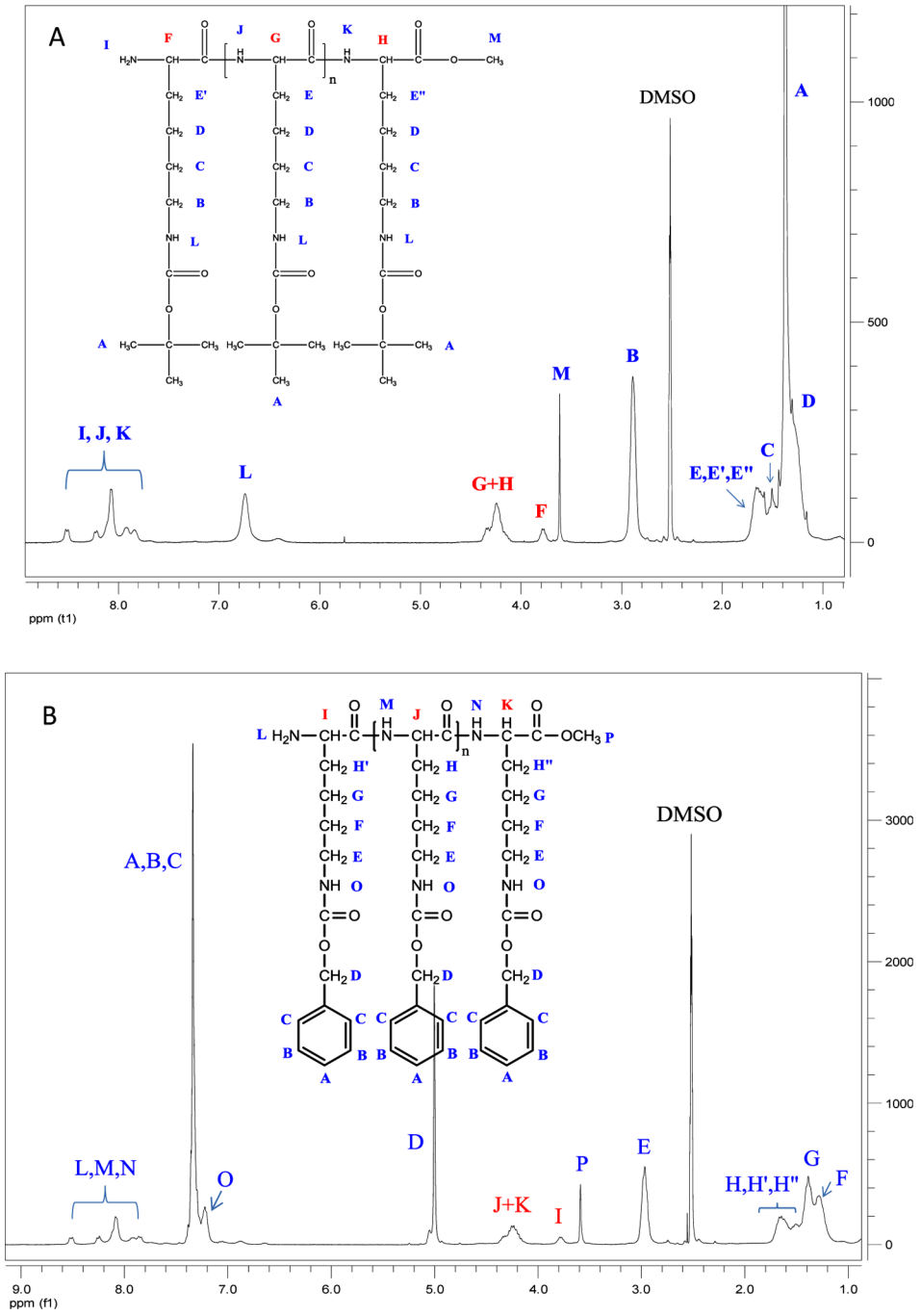

Figure 2. ^{1}H NMR (300 MHz, DMSO-d_6) spectra of (A) oligo(N_ε-Boc-L-lys) and (B) oligo(N_ε-Z-L-lys). Oligomerizations were conducted by papain (0.54 mg/mL) at pH 7 (pH stat controlled) and 40 °C for 3 h.

papain-catalyzed oligo(N_ε-Boc-L-lys) synthesis was determined, and the results are plotted in Figure 3A. A maximum %-yield of oligo(N_ε-Boc-L-lys) was obtained at pH values of 6.5 to 7.5. This pH range agrees well with previous studies on papain-catalyzed oligomerizations of other amino acid esters such as glutamate diethyl ester and lysine ethyl ester.[17,21a] From pH 6.5 to 6.0, the %-yield decreases from 35 ± 7 to 22 ± 7. Also, increase in the pH from 7.5 to 8 resulted in a decrease in %-yield from 33 ± 1 to 18 ± 8. In terms of the DP_{avg}, the maximum DP_{avg} (7.5 ± 0.1) was obtained at pH 6; from pH 6.5 to 7.5 the DP_{avg} remains at about 5.7, while at pH 8 the DP_{avg} reached its lowest value (4.7 ± 0.2). Hence, by decreasing the pH at which L-lys[Boc] oligomerizations were preformed from 8 to 6, an increase in DP_{avg} from 4.7 ± 0.2 to 7.5 ± 0.1 was attained. However, to achieve maximum %-yields one must compromise on the product DP_{avg}. A possible explanation is that the lower reaction pH will enhance the solubility of the oligomers, which is one of the major factors determining the oligomer molecular weight.

The pH dependence for papain-catalyzed oligo(N_ε-Z-L-Lys) synthesis was determined and the results are plotted in Figure 3B. There is no significant change in oligo(N_ε-Z-L-Lys) %-yield, which remained at about 20% for reactions conducted at pH values of 6, 6.5, 7.0, 7.5, and 8.0. In contrast, for oligo(N_ε-Z-L-Lys) synthesis, the %-yield plateau is at about 34% for reactions conducted at pH values of 6.5, 7.0, and 7.5. As the pH at which

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Table 1. Comparison of %-Yield, DP_{avg}, M_n, and $Đ_M$ of Oligo(L-Lys), Oligo(N_ε-Boc-L-lys), and oligo(N_ε-Z-L-lys)[a,b]

substrate methyl ester	yield (%)	DP_{avg}[e]	DP_{avg}[f]	$Đ_M$[e]
Lys[c]	23		2.7	1.1
Lys[Boc][d]	34	5.8	5.6	1.02
Lys[Z][d]	24	4.9	4.1	1.01

[a]Oligomerizations were performed by papain catalysis at pH 7 (pH stat controlled) and 40 °C for 3 h. [b]Product analysis by HPLC-UV was performed after deprotection of the N_ε-Boc and N_ε-Z moieties. [c] %-yield, DP_{avg} by HPLC-UV, M_n, and $Đ_M$ are calculated based on analysis of products in the media as described in ref 17. [d]%-yield, DP_{avg}, and $Đ_M$ are calculated on the product that precipitates from the reaction medium. [e]Calculated by HPLC-UV. [f]Calculated by ^{1}H NMR.[17]

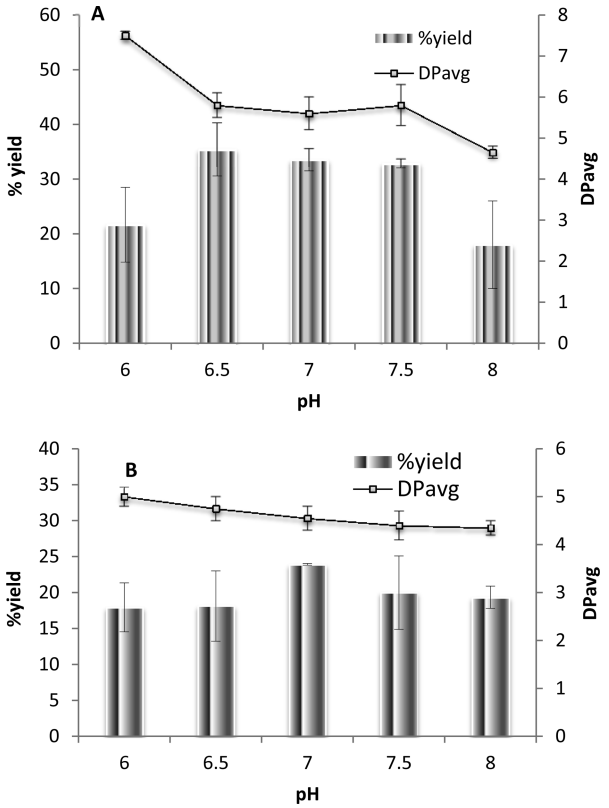

Figure 3. Effect of reaction pH on %-yield and DP_{avg} values for the preparation of (A) oligo(N_ε-Boc-L-Lys) and (B) oligo(N_ε-Z-L-Lys). Oligomerizations were conducted with 0.5 M monomer and 0.54 mg/mL papain, at 40 °C, for 3 h. ^{1}H NMR was used to determine the DP_{avg}. Error bars represent the deviation from the mean of duplicate experiments.

N_ε-Boc-L-Lys-OMe oligomerizations were preformed was decreased, the DP_{avg} increased slowly from 4.3 ± 0.2 to 5 ± 0.2. From above, we see that oligo(N_ε-Boc-L-Lys) DP_{avg} also increased as the pH at which the reaction was performed decreased but the magnitude of the change was greater (4.7 ± 0.2 to 7.5 ± 0.1).

To explain the above differences in %-yield and DP_{avg} for the two N_ε-protected L-Lys-methyl ester monomers, it is useful to consider dissimilarities in their structures. An intuitive comparison of N_ε-Z and N_ε-Boc L-lys indicates that the former will have lower water solubility. Furthermore, the fact that the benzyloxycarbonyl moiety is larger relative to the *tert*-butoxycarbonyl group should lead to divergences in binding and kinetic constants for the corresponding papain-catalyzed oligomerizations. Hence, we believe the relatively smaller Boc groups allow chains of N_ε-Boc-L-lys units to attain relatively higher DP_{avg} values prior to their precipitation from the reaction medium. However, an understanding of the relative binding and kinetic constants for these two monomers is needed for further discussion of these results.

Effect of Enzyme Concentration. Enzyme concentrations used for the reactions described in the preceding sections were based on literature values from previous studies on papain-catalyzed oligo(γ-Et-Glu), oligo(L-Phe), and oligo(L-Lys) syntheses.[17,21a,b] Figure 4 displays the effect of papain

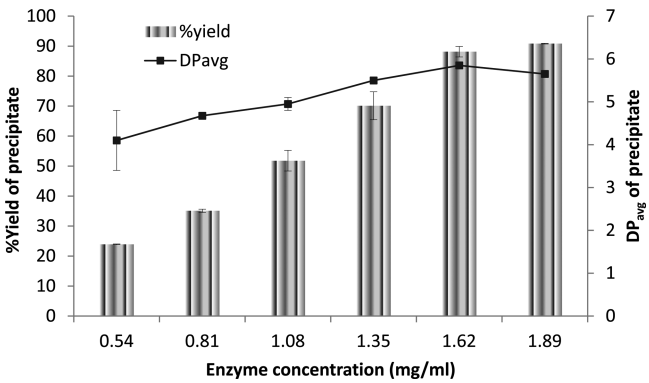

Figure 4. Effect of papain concentration on oligo(N_ε-Z-L-lys-OMe) %-yield and DP_{avg}. Reactions were conducted at 40 °C for 3 h, with monomer concentration fixed at 0.5 M. Error bars represent the deviation from the mean of duplicate experiments.

concentration on the %-yield and DP_{avg} of oligo(N_ε-Z-L-lys). By increasing the papain concentration from 0.54 to 1.62 mg/mL, the %-yield of oligo(N_ε-Z-L-lys) steadily increases from 24 ± 0 to 88 ± 2. Concurrently, the product DP_{avg} increases from 4.1 ± 0.7 to 5.7 ± 0.1. Further increase in the papain concentration from 1.62 to 1.89 mg/mL results in no substantial change in the %-yield and a small decrease in the DP_{avg} (5.9 ± 0.1 to 5.7 ± 0.1). The %-yield of 88 ± 2 is larger than literature values reported by us and others for oligopeptide syntheses from various protease-amino acid alkyl ester combinations.[17,21a,b,d,e,32,33]

An increase in the catalyst concentration with a constant monomer concentration should lead to an increase in the concentration of activated enzyme−substrate ($E{\cdot}S_{N\varepsilon\text{-Z-L-lys}}$) species and, therefore, %-monomer conversion to oligomer. Also, one would expect that increased enzyme concentration would also result in the formation of a larger number of chains leading to a lower DP_{avg}. From the results above it appears that product hydrolysis occurs at a slower rate than oligomer synthesis. Furthermore, even if short chain fragments are initially formed at high enzyme concentrations, these short chains can grow by condensation reactions between oligomers leading to higher chain length oligomers that precipitate from the reaction medium. The need for higher enzyme concentrations is likely due to slow propagation reactions of N_ε-Z-L-lys-OMe and corresponding oligomers which are non-natural substrates.

Oligo(N_ε−Z−L-Lys-OMe) Synthesis As a Function of Reaction Time. Figure 5A displays the %-yield of oligo(N_ε-Z-L-lys) as a function of reaction time. Oligomerizations were conducted with 0.5 M substrate concentration, in phosphate buffer (0.45M, pH = 7) at 0.54 mg/mL (black) and 1.89 mg/

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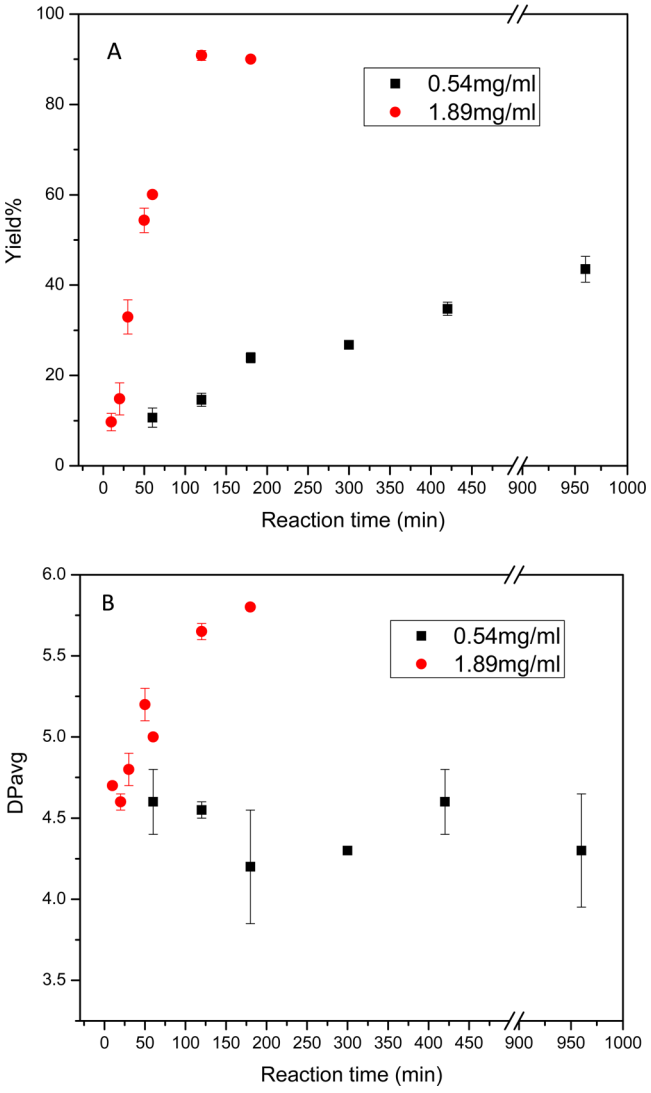

Figure 5. Effect of reaction time on oligo(N_ε-Z-L-lys) (A) %-yield and (B) DP_{avg}. Reactions were conducted using either 0.54 or 1.89 mg/mL in 0.45 M phosphate buffer (pH = 7, pH stat controlled) with monomer concentration fixed at 0.5 M. Error bars represent the deviation from the mean of duplicate experiments.

mL (red), papain concentrations, respectively. With 0.54 mg/mL papain, the oligo(N_ε-Z-L-lys) %-yield increased slowly from 11 ± 2 at 1 h to 24% in 3 h. In contrast, from 1 to 3 h, the DP_{avg} showed no significant change and remained at about 4.3. Since oligomerizations at 0.54 mg/mL proceeded slowly, reactions were performed for time periods up to 16 h. The %-yield continued to increase steadily until 16 h reaching 44 ± 3% in 16 h. However, from 3 to 16 h, oligo(N_ε-Z-L-lys) DP_{avg} values remained at about 4.3. Since oligo(N_ε-Z-L-lys) yields reached 88 ± 2% within 3 h at 1.89 mg/mL catalyst, the progression of product formation as a function of reaction time was also studied at this concentration. Indeed, %-yield with time values increased rapidly at 1.89 mg/mL catalyst reaching 32, 54, and 91% at 30 min, 50 min, and 2 h, respectively. Figure 5B shows that, at 1.89 mg/mL catalyst concentration, the DP_{avg} also follows the same trend as %-yield by steadily increasing as a function of reaction time. From reaction times of 30 and 50 min and 2 and 3 h, DP_{avg} values are 4.8, 5.2, 5.7, and 5.8.

Influence of Water Miscible Cosolvents on Oligo(N_ε-Z-L-lys) Synthesis. The use of water miscible cosolvents provides an additional tool for engineering the reaction conditions to enhance desired product formation.[13d,21b,c,e,34] For example, the addition of water-miscible cosolvents can increase the solubility of hydrophobic amino acid monomers that can result in higher oligopeptide yields.[14a,21b,c] It is also well-known that the choice of water-miscible organic solvents and its concentration relative to water can result in decreased enzyme stability and activity.[13d,14a,34b] Given this information, identification of one or more potentially beneficial water-miscible organic solvents that can increase the %-yield and/or DP_{avg} of oligo(N_ε-Z-L-lys) was explored.

Figure 6 illustrates the effect of different cosolvents on the %-yield and DP_{avg} of oligo(N_ε-Z-L-lys) prepared by papain

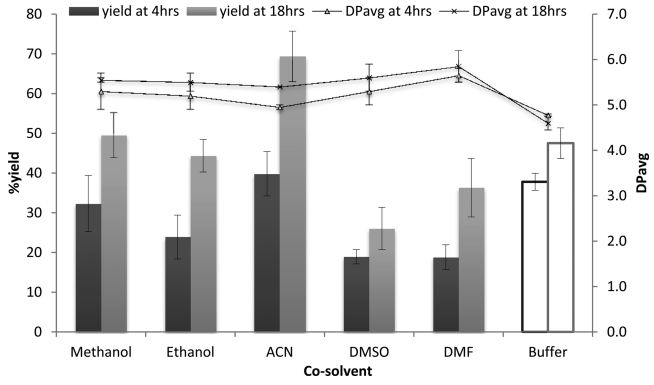

Figure 6. Synthesis of oligo(N_ε-Z-L-lys) in solutions of 80% v/v aqueous phosphate buffer (0.45 M, pH = 7) and 20% v/v water-miscible cosolvents (MeOH, EtOH, ACN, DMSO, DMF). All reactions were conducted with 0.5 M N_ε-Z-L-lys-OMe, at pH 7, for 4 and 18 h, using 0.81 mg/mL papain as the catalyst.

catalysis. Since extending the reactions beyond 3 h was found to have beneficial effects on oligo(N_ε-Z-L-lys) %-yield (Figure 5A), reactions were performed for 4 and 18 h. The cosolvents investigated include methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), dimethyl sulfoxide (DMSO), and dimethylformamide (DMF). These solvents were selected since they were all found to improve the %-yield relative to pure phosphate buffer (0.25 M) of papain-catalyzed oligo(L-Phe) synthesis from L-Phe-OEt.[21b] Furthermore, based on ref 21b, the ratio of organic solvent-to-aqueous phosphate buffer (0.45 M, pH = 7) was fixed at 20/80 (%-v/v). The %-yield of oligo(N_ε-Z-L-lys) in pure buffer as well as with all the cosolvents increased as the reaction time was extended from 4 to 18 h. Comparison of %-yields at 4 h shows that the %-yield was about 40% both in pure buffer and by using ACN as the cosolvent. However, for all of the other cosolvent-buffer solutions, the %-yield at 4 h was lower than in pure buffer. Hence, except for ACN, buffer-cosolvent mixtures appear to decrease the propagation rate for conversion of N_ε-Z-L-lys-OMe to precipitated oligo(N_ε-Z-L-lys).

Small but significant increases in DP_{avg} values were observed for 4 h reactions conducted in cosolvent-buffer solutions instead of in pure buffer (Figure 6). The DP_{avg} increased from about 4.8 in pure buffer to 5.2 in aqueous buffer solutions containing 20%-by-vol of either MeOH, EtOH, or DMSO. In DMF-buffer solution, the DP_{avg} reached 5.7. Higher DP_{avg} values in cosolvent-buffer solutions are attributed to increased oligopeptide solubility. Figure 6 shows that, at 18 h, %-yields in

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MeOH and EtOH-buffer solutions were equivalent to that in pure buffer (about 47%). However, in ACN-buffer solution, the %-yield is 69 ± 6%. DP_{avg} values for 4 and 18 h reactions in solvent-buffer and pure buffer reaction media were unchanged with the exception of ACN-buffer solution where the DP_{avg} increased from 5.0 to 5.4.

CONCLUSION

The ability of papain to convert N_ε-Boc-L-lys-OMe to oligopeptides with DP_{avg} 7.5 ± 0.1 at pH 6 demonstrated that papain subsites can accommodate the corresponding large hydrophobic peptide side chains during propagation reactions. Analysis of products by ^{1}H NMR and HPLC-UV-MS showed that oligo(N_ε-Boc-L-Lys) and oligo(N_ε-Z-L-Lys) were synthesized with one free amino group that resides at the oligomer N-terminal position. This will be useful in future work where we plan to selectively conjugate N_ε-protected oligo(L-lys) to nanoparticles, polymer chains, surfaces, and fibers after which N_ε-protecting groups will be removed.

Decreasing the pH at which oligomerizations were performed from 8 to 6 had a greater effect on the DP_{avg} of oligo(N_ε-Boc-L-Lys) than oligo(N_ε-Z-L-Lys). For oligo(N_ε-Boc-L-Lys), the DP_{avg} increased from 4.7 ± 0.2 to 7.5 ± 0.1 by conducting the reaction at the more acidic pH where oligomeric products are more soluble. Unfortunately, maximum yields and DP_{avg} do not share the same pH optimum values. Hence, increasing the activity of papain at lower pH through protein engineering would be an effective route to improving oligomer synthesis efficiency as well as increasing product chain lengths.

Increase in the papain concentration was an effective way to increase the oligo(N_ε-Z-L-lys) yield and average chain length. By increasing the papain concentration from 0.54 to 1.62 mg/mL, the %-yield and DP_{avg} of oligo(N_ε-Z-L-lys) steadily increases from 24 ± 0 to 88 ± 2 and 4.1 ± 0.7 to 5.7 ± 0.1, respectively. The %-yield of 88 ± 2 is larger than literature values reported by us and others for oligopeptide syntheses from various protease-amino acid alkyl ester combinations.

ASSOCIATED CONTENT

Supporting Information

Figure S1: ^{1}H NMR spectrum of N_ε-Boc-L-lys-OMe. Figure S2: ^{1}H NMR spectrum of N_ε-Z-L-lys-OMe. Figure S3: SDS PAGE analysis of crude papain. Method for determination of papain content in the crude enzyme extract. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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