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Protease-Catalyzed Oligomerization of L-Lysine Ethyl Ester in Aqueous Solution

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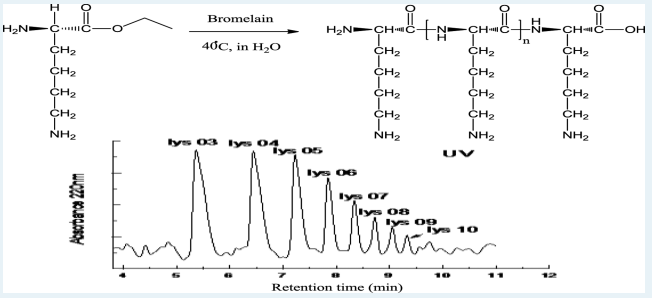
Supporting Information

ABSTRACT: This paper describes the protease-catalyzed synthesis of oligo(L-Lys) from L-Lys ethyl ester (L-Lys-Et) in an aqueous reaction medium at controlled pH using a pH-stat. Four proteases (papain, bromelain, α-chymotrypsin, and trypsin) were studied to determine their activity for L-Lys ethyl ester oligomerization at pH values ranging from 6 to 11. Bromelain was found to be preferred relative to the other protease catalysts because it gave the highest values of oligo(L-Lys) yield and optimal average chain length (DP_{avg}). To evaluate reaction progress and product structure, ^{1}H NMR and HPLC−UV-MS methods were developed. A series of model oligo(L-Lys) compounds with chain lengths 2, 3, and 4 was obtained and analyzed to assign ^{1}H NMR signals. All components of the mixture were successfully resolved and analyzed by HPLC−UV-MS by ion-pairing reversed-phase chromatography using heptafluorobutyric acid as the ion-pairing agent. The effects of medium pH, L-Lys-Et concentration, bromelain concentration, reaction time, and reaction temperature on oligo(L-Lys) synthesis was evaluated. Oligomers with chain lengths more than 10 are formed within 5 min. By 30 min, the DP_{avg} and longest oligomer chain lengths reached maximum values of ∼3.6 and 12.0, respectively. Residual L-Lys-Et was only 2% by 40 min. Products formed by 30 min remained relatively unchanged as the reaction was continued for 2.5 h. The competitive reaction pathways during oligomer initiation, propagation and transamidation/hydrolysis pathways are presented and discussed relative to the results obtained herein.



KEYWORDS: oligo(lysine), protease catalysis, peptide synthesis, bromelain, HPLC

INTRODUCTION

During the past two decades, peptides have been extensively investigated in many fields of importance, notably for (i) novel hydrogel scaffolds,[1,2] (ii) surfactants,[2] (iii) cosmetic ingredients,[3] (iv) biologically active peptides,[4] and (v) gene delivery vehicles.[5] Most often, the preparation of peptides with a precise sequence of amino acids is performed by solid phase (SPPS) or liquid phase (LPPS) peptide synthesis methods.[6,7] Although these approaches provide peptides with uniform chain lengths and sequences, they are costly, since they require use of protection−deprotection chemistry, toxic reagents, and product purification. Protein engineering of desired peptide sequences into organisms and subsequent production of peptides via fermentation provides an alternative route to prepare precise sequence and chain length products. Although this approach is promising, peptide yields via fermentation are generally low, and diversification of peptide structure via incorporation of non-natural amino acids requires specialized methods.[8,9] As a result, although it is tempting to consider expanding the range of applications for which peptides are currently used, the high cost of these methods for peptide synthesis becomes limiting. Therefore, there is a need for new peptide synthetic methods that are scalable, safe, and cost-effective. Protease-catalyzed peptide synthesis is of interest in this regard, since it circumvents the aforementioned difficulties associated with conventional synthetic approaches discussed above. This paper is focused on the use of the serine and cysteine proteases, belonging to the family of endopeptidases, for oligomerization of L-Lys-Et, in which both the substrate and formed oligomers are soluble in the reaction medium.

Although the natural role of proteases is catalysis of peptide hydrolysis, by proper manipulation of the physiological conditions of a given reaction, proteases can efficiently catalyze peptide bond-forming reactions.[10,11] Peptide bond synthesis by protease catalysis is generally accomplished by either thermodynamically or kinetically controlled reaction conditions.[11] In kinetically controlled synthesis, −OH or −SH at active sites of serine or cysteine proteases undergoes a nucleophilic attack at the substrate carbonyl carbon to form an acyl-enzyme intermediate.

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The deacylation of the acyl-enzyme intermediate by the amino group of an amino acid substrate to form a peptide bond is called aminolysis, whereas deacylation by reaction with water results in hydrolysis. Hence, hydrolysis and aminolysis deacylation pathways occur competitively.[12]

A challenge in developing kinetically controlled peptide synthesis reactions is to end reactions when the desired kinetic product is obtained. Otherwise, the reaction will continue to form the equilibrium product. For protease-catalyzed L-Lys-Et oligomerization, in which both the substrate and oligo(L-Lys) products are soluble in the reaction medium, moving from the kinetic to the equilibrium product implies hydrolysis of oligo(L-Lys) to shorter oligomers, resulting over time in L-lysine.[11] This paper describes how critical reaction parameters, such as protease activity, protease concentration, substrate concentration, pH, and reaction time, influence the conversion of L-Lys-Et to oligo(L-Lys) so that formation of the kinetic product with an optimal average chain length (DP_{avg}) is achieved.

A summary of important progress in protease-catalyzed routes to synthesize oligopeptides is available in recent publications by us and others.[13−15] The discussion below provides historical perspective and a brief synopsis of relevant literature. Oligopeptides of Leu, Met, Phe, and Tyr with DP < 10 were successfully synthesized in 1979 and 1980 by Luisi and co-workers.[17,18] Thereafter, Aso and co-workers[16,19] reported the protease-catalyzed synthesis of oligo-L-glutamic acid from L-glutamic acid diethyl ester in both phosphate buffer and organic phase. Papain catalysis in buffer of methionine, phenylalanine, threonine, and tyrosine ester hydrochlorides gave poly(α-amino acid)s with DP_{avg} < 10.[20−22] Oligomerization of L-tyrosine ethyl ester (L-Tyr) by papain catalysis resulted in products with chain lengths from 4 to 9 tyrosine units.[23] In addition, these workers recorded $^1H-^1H$ COSY NMR spectra of oligo(Et-L-glu) prepared by protease catalysis and concluded that oligomers formed consist exclusively of α-linked γ-ethyl glutamate units.

Soeda et al.[22] reported that oligomerization of diethyl L-aspartate at 40 °C for 2 days in MeCN containing 4.5% (v/v) water gave α-linked poly(β-ethyl L-aspartate) in 85% yield and M_w up to 3700. Li et al.[24] found that, after only 15 min reactions, L-Et_2-Glu was converted to oligo(γ-L-Et-Glu) in yields of ∼80%. Li et al.[25] also studied co-oligomerizations of L-leucine ethyl ester (L-Et-Leu) and Et_2-L-Glu. The relative activity of protease catalysts for this co-oligomerization was as follows: papain ≈ bromelain > α-chymotrypsin > protease SG. Furthermore, characterization of products by multiple methods showed that all four proteases have no apparent specificity with respect to a preference for adding either L-Et-Leu to a L-Et-Glu terminal propagating chain or γ-L-$(Et)_2$-Glu to a LLeu terminal unit.

Viswanathan et al.[15] developed a one-pot biotransformation to synthesize oligo(γ-L-Et-Glu) decorated with selected amine-functionalized end-groups at C-termini. Irrespective of the protease used, 2-thiophene methyl amine gave the highest fraction of oligo(γ-L-Et-Glu)-NH-R chains. Viswanathan et al.[14] also studied enzymatic synthesis of oligopeptides from hydrophobic amino acid ethyl esters.[14] By using L-phenylalanine as a model system, the effect of different proteases, water miscible cosolvents, and different ratios of water-miscible cosolvents on the protease-catalyzed oligopeptide were compared.

Oligo(L-Lys) alone or conjugated with a lipid, PEG or hydrophobic amino acid oligomer, is of intense interest because of the wide range of important applications for which oligo(L-Lys)-containing materials can be employed. Examples include uses of oligo(L-Lys) containing materials for (i) self-assembling amphiphilic nanostructures that form hydrogel scaffolds,[26] (ii) acid-sensitive micelles for drug delivery,[27] (iii) oligo(L-Lys)-based oligosaccharide clusters for cell-specific intercellular adhesion,[28] (iv) photoresponsive dendritic peptides,[29] (v) intelligent nanobiomaterials for hyperthermic gene delivery,[30] (vi) polyelectrolytes,[31] and more. The approach to synthesize L-Lys-containing peptides has relied on SPPS, LPPS, and ring-opening polymerization (ROP) of α-amino acid *N*-carboxylic anhydrides (NCAs). The inefficiency of SPPS and LPPS, due to protection/deprotection and coupling steps and the use of toxic solvents leads to high-cost processes that are unsuitable for applications outside of therapeutics and specialty cosmetic products.[4] ROP of NCA monomers requires toxic phosgene or phosgene equivalents for NCA synthesis, and NCA ROP requires strict removal of water and high monomer purity.

Whereas water-insoluble oligomers consisting of nonpolar amino acids have been prepared with papain, α-chymotrypsin, and other proteases in aqueous reaction media, protease-catalyzed synthesis of cationic peptides from basic amino acids in aqueous medium was described once in a pioneering study in 1992 by Aso and Kodaka.[32] These workers investigated trypsin-catalyzed oligomerization of L-Lys-Et in sodium carbonate buffer. By using HPLC with online ninhydrin derivatization to measure free amino acid in products, they revealed how the yield and initial reaction rate (V_{app}) of trypsin-catalyzed conversion of L-Lys-Et to oligo(L-Lys) is influenced by medium pH, reaction time, and other parameters. An overall reaction yield higher than 70% was attained after 2 h at pH 10. Although Aso and Kodaka[32] demonstrated the potential of preparing oligo(L-Lys) by protease catalysis, it would have been useful if they had established methods to quantify DP_{avg}, chain length distribution, and formation of free L-Lys (hydrolysis instead of amidation reactions). Such information could be used to interrogate the relative importance of competing reactions during oligomer formation. In addition, it may be that other proteases are more effective than trypsin for oligo(L-Lys) synthesis.

In this paper, we expand the proteases studied for oligo(L-Lys) synthesis by including papain, bromelain, and α-chymotrypsin in addition to trypsin. Of these catalysts, bromelain was found to be most efficient, giving the highest percent monomer conversion and oligo(L-Lys) DP_{avg} values. Further studies evaluated the effects of substrate concentration, enzyme concentration, reaction pH, reaction time, and reaction temperature on percent monomer conversion and DP_{avg} values. This work resulted in identification of reaction conditions that give monomer percent conversion of close to 90%, longest chain lengths up to 12 units, and DP_{avg} of ∼4. By assignment of peaks using uniform chain length oligo(L-Lys) standards, ^1H NMR was found to be an effective tool for determining both percent conversion and DP_{avg} values of oligo(L-Lys) mixtures. For comparative purposes, percent monomer conversion values were also measured by HPLC−UV-MS via quantification of nonoligomerized L-Lys and L-Lys-Et, and oligo(L-Lys) DP_{avg} was determined from relative intensity of LC peaks corresponding to oligo(L-Lys) of different chain lengths. Insights into the relative importance of competing reactions during oligomer formation are discussed.

EXPERIMENTAL SECTION

Materials. L-Lys-Et dihydrochloride was purchased from Sigma in the highest available purity and was used as received.

Crude papain (EC no. 3.4.22.2; source-Carica papaya; 30 000 USP units/mg of solid; molecular weight 21K) was purchased from CalBioChem. Bromelain (cysteine protease; EC 3.4.22.4; source, pineapple stem; 2.290 units/mg solid; 3.650 units/mg protein), α-chymotrypsin (serine protease; EC 232-671-2; source, bovine pancreas, type II, 83.9 units/mg solid; 96 units/mg protein), trypsin (serine protease; EC 232-650-8; source, bovine pancreas) were purchased from Sigma Aldrich Inc. and were used as received.

Methods. *General Procedure Followed for Protease Catalyzed Oligo(L-Lys) Synthesis.* L-Lys-Et hydrochloride (494.4 mg, 2 mmol), and 4 mL of water or phosphate buffer solution were transferred to a 15 mL Erlenmeyer flask. A Tiamo automatic titration system and a Metrohm CH9101 dosing units were used to control the pH of the reaction media within ±0.05 units of the set value during oligomerizations. The dosing solution (3 M NaOH) was added at 0.05−0.1 μL/min, and the frequency at which the probe checked the pH was set to 1.0 s. After pretitration manually or by the pH stat to set reactions at the desired pH, a predetermined amount of protease was added. 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. After reactions, protease was 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 two days to give a solid.

Chemical Hydrolysis L-Lys-Et As a Function of pH. L-Lys-Et hydrochloride (494.4 mg, 2 mmol), and 4 mL of water or phosphate buffer solution were transferred to a 15 mL Erlenmeyer flask. As above, a Tiamo automatic titration system was used with identical parameter set values. After pretitration manually or by the pH stat to set reactions at the desired pH, 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. Products were lyophilized and then further characterized by ^{1}H NMR and LC−MS.

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 were performed in DMSO-d_6/TFA-d cosolvent (volume 30:1) at 10 mg/mL with a data acquisition delay of 1 s and a total of 128 scans. Data were collected and analyzed by MestRe-C software. Proton chemical shifts were referenced to tetramethylsilane (TMS) at 0.00 pm.

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 electron spray 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.

After reactions, the protease was removed from the peptide mixture by ultracentrifuge using a membrane with 3000 molecular weight cutoff. The mobile phase consisted of three eluents: eluent A, water; eluent B, acetonitrile; elutent C, 1% (v/v in water) heptafluorobutyric acid (HFBA). For oligolysine analysis, the separation started with 80% A, 10% B, and 10% C. The composition changed to 45% A, 45% B, and 10% C in a linear gradient at a flow rate of 1 mL/min. Eluate from the column was split into two parts. One part was analyzed by UV detection, and the other was analyzed in full scan mode by MS detection. The UV and MS data were collected and analyzed by Micromass Masslynx 4.0 software.

For quantification of L-Lys and L-Lys-Et, standards of these compounds were prepared in 0.1% (v/v in water) HFBA with 0.3 mg/mL NaCl. This NaCl concentration is equivalent to the NaCl content of samples analyzed. L-Lys and L-Lys-Et standards with concentrations 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, and 0.5 mg/mL were injected onto the column. Analysis of L-Lys was performed in isocratic mode using 80% A, 10% B, and 10% C and the Lys peak was detected at m/z = 147 in single ion record mode by MS detection. Analysis of L-Lys-Et was performed using 75% A, 15% B, and 10% C, and the peak of L-Lys-Et was detected at m/z = 175 in single ion record mode. Calibration curves were constructed by plotting peak area of L-Lys or L-Lys-Et vs concentration.

RESULTS AND DISCUSSION

On the basis of previous studies describing the kinetic model for serine- or cysteine-type protease-catalyzed peptide synthesis,[10,11] the mechanism in Scheme 1 describes the sequence of reactions that are believed to occur during conversion of L-Lys-Et to oligo(L-Lys). Scheme 1A illustrates steps involved during initiation of oligomer synthesis. This comprises reaction between L-Lys-Et and the protease (E-XH) to form an enzyme−substrate tetrahedral intermediate that is converted to the corresponding enzyme-activated monomer (acyl-enzyme) complex, En−X-L-Lys−NH_2.[10,11] Thereafter, this complex is deacylated by reaction with L-Lys-Et to give NH_2−Lys-Lys-Et.

Water can also act as a competitive initiator and can form to various extents hydrolyzed free L-Lys. Indeed, the extent that deesterified L-Lys accumulates in reaction media is discussed below. Scheme 1B illustrates steps involved in further propagation of NH_2−Lys-Lys-Et or, more generically, NH_2−$(Lys)_m$-Et, to higher oligomers. Similar to Scheme 1A, NH_2−$(Lys)_m$-Et is converted to the corresponding acyl-enzyme complex, NH_2−L-$(Lys)_m$-X-En. Thereafter, this complex can be deacylated with amine acyl acceptors L-Lys-Et or higher oligomers (NH_2−[L-Lys]$_n$-Et) to give NH_2−$(Lys)_m$-$(Lys)_n$-Et. Furthermore, any of the above acyl enzyme complexes may be deacylated by water, resulting in an oligomer with a terminal free carboxylic acid (Y = H) that is deactivated from further propagation reactions as an acyl donor but that can still function as an acyl acceptor. The relative extent that oligo(L-Lys) is formed by chain-type reactions in which subsequent additions of L-Lys-Et to oligomer chain ends occurs one unit at a time, versus step-type condensation oligomerizations in which oligomers are primarily formed by reactions between chain segments, such as NH_2−[L-Lys]$_m$-X-En and NH_2−[L-Lys]$_n$-Et, is currently unknown. Furthermore, the average chain length of oligomers, DP_{avg}, determined as a function of experimental variables discussed below, will ultimately be a function of how these variables alter the relative rates of propagation or aminolysis relative to deacylation by reactions with H_2O.

In contrast to the study herein, previous work by us and others[14,20,23−25] largely focused on protease-catalyzed oligomerizations of L-amino acid esters that, when a certain chain length is reached, become insoluble and precipitate from the reaction medium. In these cases, chain length is largely determined by oligomer solubility, whereas, for oligo(L-Lys), oligomers remain in solution. To reach relatively higher DP_{avg} values, in addition to the requirement that the protease recognizes relatively higher chain length oligo(L-Lys) as a substrate for further propagation reactions, a further requirement is to maintain a high fraction of

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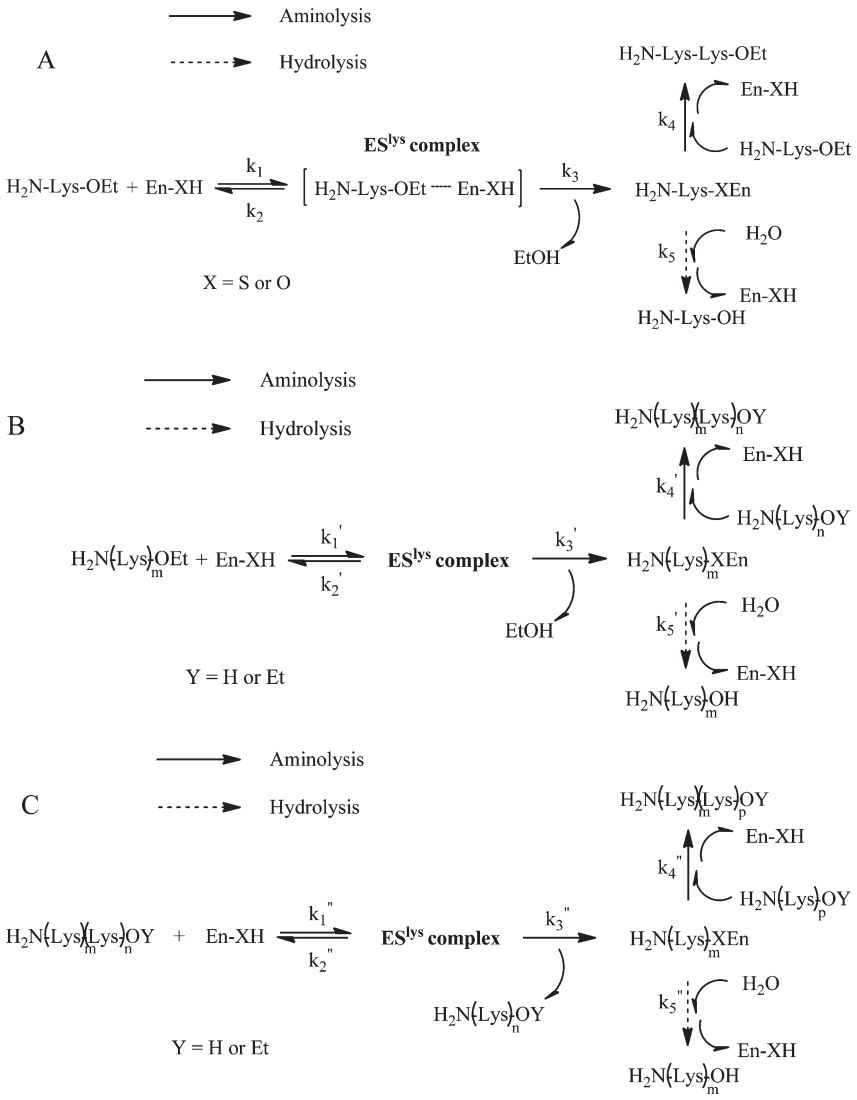
Scheme 1. Reaction Mechanism Based on the Kinetic Model of Protease-Catalyzed Amino Acid Ester Oligomerization[a]



[a] (A) Initiation step, (B) propagation steps to form oligo(L-Lys), and (C) hydrolysis reactions leading to oligomer degradation.

terminal ethyl ester moieties. Hence, a desirable characteristic of a protease catalyst is to prefer amidation reactions over hydrolysis reactions. Another possible competing reaction is formation of $NH_2-(L\text{-Lys})_m$-X-En, in which the acyl donor is an internal amide bond, such as that between $NH_2-(Lys)_m-$ and $-(Lys)_n-OY$ segments of $NH_2-(Lys)_m-(Lys)_n-OY$. As depicted in Scheme 1C, H_2O can function as the acyl acceptor, leading to products $NH_2-(Lys)_m-OH$ and $NH_2-(Lys)_n-OY$. Alternatively, the protease can catalyze a transamidation reaction in which the acyl acceptor is $NH_2-(Lys)_p-OY$, resulting in the synthesis of $NH_2-(Lys)_m-(Lys)_p-OY$.

To increase the relative rate of propagation for oligo(L-Lys) synthesis versus reactions that would result in lower DP_{avg} oligomers (see above), systematic investigations into factors that can influence oligo(L-Lys) percent yield and DP_{avg} are described below. These variables include the protease used, reaction pH, substrate concentration, enzyme concentration, reaction time, and temperature. Furthermore, methods developed to better characterize products are discussed in detail below.

STRUCTURAL ANALYSIS

HPLC–UV-MS Analysis of Oligolysine. Oligo(L-Lys) carries multiple charges at both acidic and neutral pH, which makes it hydrophilic at commonly used reversed-phase conditions. Therefore, oligo(L-Lys) is poorly retained by the reversed-phase column. To solve this problem, the ion-pairing reagent heptafluorobutyric acid (HFBA) was added to the mobile phase. The HFBA anions not only neutralize oligo(L-Lys)'s positive charges but also increase its hydrophobicity by binding to the positive charged amine groups. The other reason HFBA was selected as the ion-pairing reagent in this study is its MS compatibility. The concept of using ion-pairing reagents such as HFBA to resolve basic peptides in reversed-phase chromatography has been described previously by others.[33−35] However, the novel feature here is that the volatility of HFBA combined with MS detection that enabled the identification of the chain length of every component of the oligo(L-Lys) mixture directly without the need to use synthetic olgolysine standards.

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Figure 1 displays the UV (220 nm) and MS chromatograms of the oligo(L-Lys) synthesized by bromelain catalysis (30 mg/mL) from 0.5 M L-Et-Lys·HCl at pH 8 and 40 °C for 1.5 h. The molecular weight of each peak was confirmed by MS detection in full scan mode. Inspection of the MS chromatogram shows the analyte is, indeed, a mixture of oligo(L-Lys) chains that consist of components with lengths from 2 to 12 units. Chromatograms recorded with UV detection show oligo(L-Lys) chains with lengths from 3 to 10 units. This discrepancy between the two chromatraphs in Figure 1 is explained by the relatively lower sensitivity of UV detection. Further details on the analysis of the oligo(L-Lys) mixture by using different concentrations of HFBA, investigations on the retention behavior of each component in the mixture, and elucidation of thermodynamic parameters involved in ion-pairing separation of oligo(L-Lys) using HFBA are given elsewhere.[36]

CALCULATION OF YIELD AND DP_{AVG} BY HPLC−UV-MS

The DP_{avg} of oligo(L-Lys) was calculated on the basis of the relative peak areas of oligo(L-Lys) constituents of differing chain lengths. 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, calculation of DP_{avg} from the UV chromatogram took into account that peak areas are influenced by both the number of peptide bonds and the concentration of each component, as is described elsewhere.[37,38] The DP_{avg} calculated on the basis of MS and UV detection is 5.0 and 4.1, respectively. It is known that the polarity of the solvent significantly influences the MS response of the analyte.[39] For example, the analytes in organic solvents such as methanol and acetonitrile have better MS sensitivity than in water.[39] This is due to the lower surface tension and higher volatility of organic solvents, which results in more efficient ionization. The percentage of acetonitrile in the mobile phase was increased as the run time progressed. Thus, relatively longer chain length components of the mixture have higher MS responses than components with fewer residues, even if they have identical concentrations. As a result, the DP_{avg} calculated on the basis of the MS analysis is overestimated. In contrast, calculation of DP_{avg} by UV detection does not show this bias because the change in the composition of the mobile phase does not influence analyte absorbance at the detection wavelength (220 nm). Further verification of the accuracy of DP_{avg} values calculated by UV detection is given below.

Calculation of Yield by HPLC− MS. Quantification of L-Lys and L-Lys-Et was performed by HPLC−MS using chromatographic conditions described in the Experimental section. While establishing conditions for the assay, it was discovered that L-Lys and L-Lys-Et peak areas varied significantly as a function of the sample salt concentrations. Hence, in the construction of standard curves, it was necessary to prepare L-Lys and L-Lys-Et in media containing a NaCl concentration identical to that of the withdrawn reaction aliquots. Calibration curves for both L-Lys and L-Lys-Et have coefficients of determination above 0.99. The yield of reactions was calculated from the quantity of L-Lys-Et that was transformed to oligo(L-Lys). Determination of [L-Lys] provided insights into the prevalence of monomer ester hydrolysis, a competitive pathway to monomer oligomerization.

^{1}H NMR Spectrum. *Calculation of Yield and DP_{avg} by ^{1}H NMR.* The ^{1}H NMR spectra of pure L-Lys, L-Lys-Et, and oligomer standards oligo(L-Lys)$_2$, oligo(L-Lys)$_3$, and oligo(L-Lys)$_4$ are displayed in Figure 2a−e, respectively. They were used as model compounds to assign internal and terminal methine signals of oligo(L-Lys) mixtures synthesized by protease catalysis. The ^{1}H NMR spectrum of a representative oligo(L-Lys) mixture, prepared by bromelain (20 mg/mL)-catalyzed oligomerization of 0.5 M L-Lys-Et at pH 7.6 (pH stat-controlled), 40 °C for 1.5 h, is displayed in Figure 3. The methine resonances of pure L-Lys, L-Lys-Et, and L-Lys oligomers (DP 2, 3, and 4) occur in the region from ∼3.8 to 4.4 ppm (Figure 2). The methine signal corresponding to C-terminal proton A shifts upfield from 3.88, for oligo(L-Lys)$_2$, to 3.82 as the oligomer chain length is increased to 3 and 4. Similarly, the methine ^{1}H NMR signal corresponding to N-terminal proton B also shifts upfield from 4.24 ppm, for oligo(L-Lys)$_2$, to 4.15 ppm, as the oligomer chain length is increased to 3 and 4. Inspection of the ^{1}H NMR for oligo(L-Lys) in Figure 3 shows that methine protons A and B are found at 3.88 and 4.19 ppm, respectively, nearby corresponding signals for model compounds oligo(L-Lys)$_3$ and oligo(L-Lys)$_4$. Further inspection of Figure 3 shows that methine protons C are found at 4.32 ppm, nearby corresponding signals for model compounds oligo(L-Lys)$_3$ and oligo(L-Lys)$_4$. Peaks H, I, and N in Figure 3 were assigned with the help of COSY-NMR (Figure S-1 of the Supporting Information).

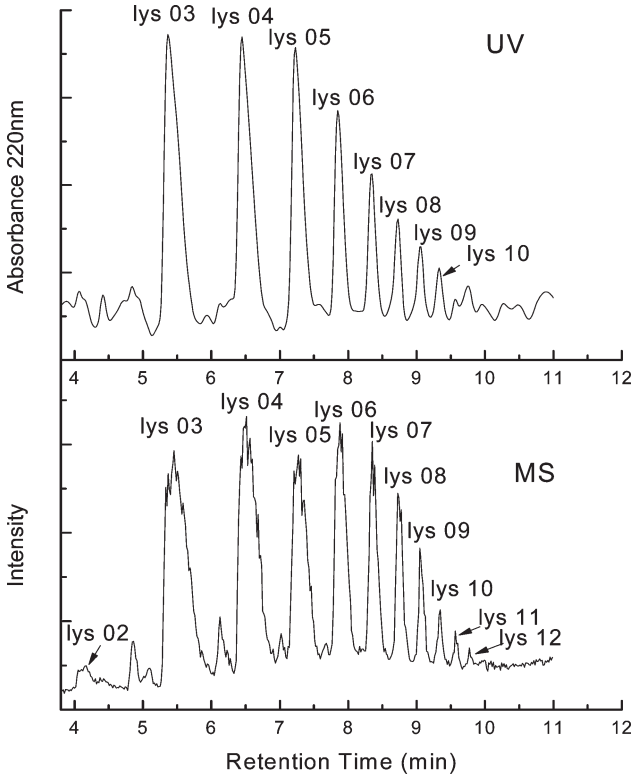

Figure 1. UV (220 nm) and MS chromatograms of oligo(L-Lys) synthesized by bromelain catalysis at pH 8, from 0.5 M L-Et-Lys·HCl, using 30 mg/mL bromelain, at 40 °C for 1.5 h.

The relative content of L-Lys, L-Lys-Et in the product mixture, percent monomer conversion to oligomers, and DP_{avg} of oligo(L-Lys) were calculated from integrations of peaks A, B·L, C, and K·M in Figure 3. B·L and K·M designate overlapped signal regions corresponding to protons B/L and K/M, respectively. Because each Lys unit of oligo(L-Lys) contains one methine group, the amount of different methine

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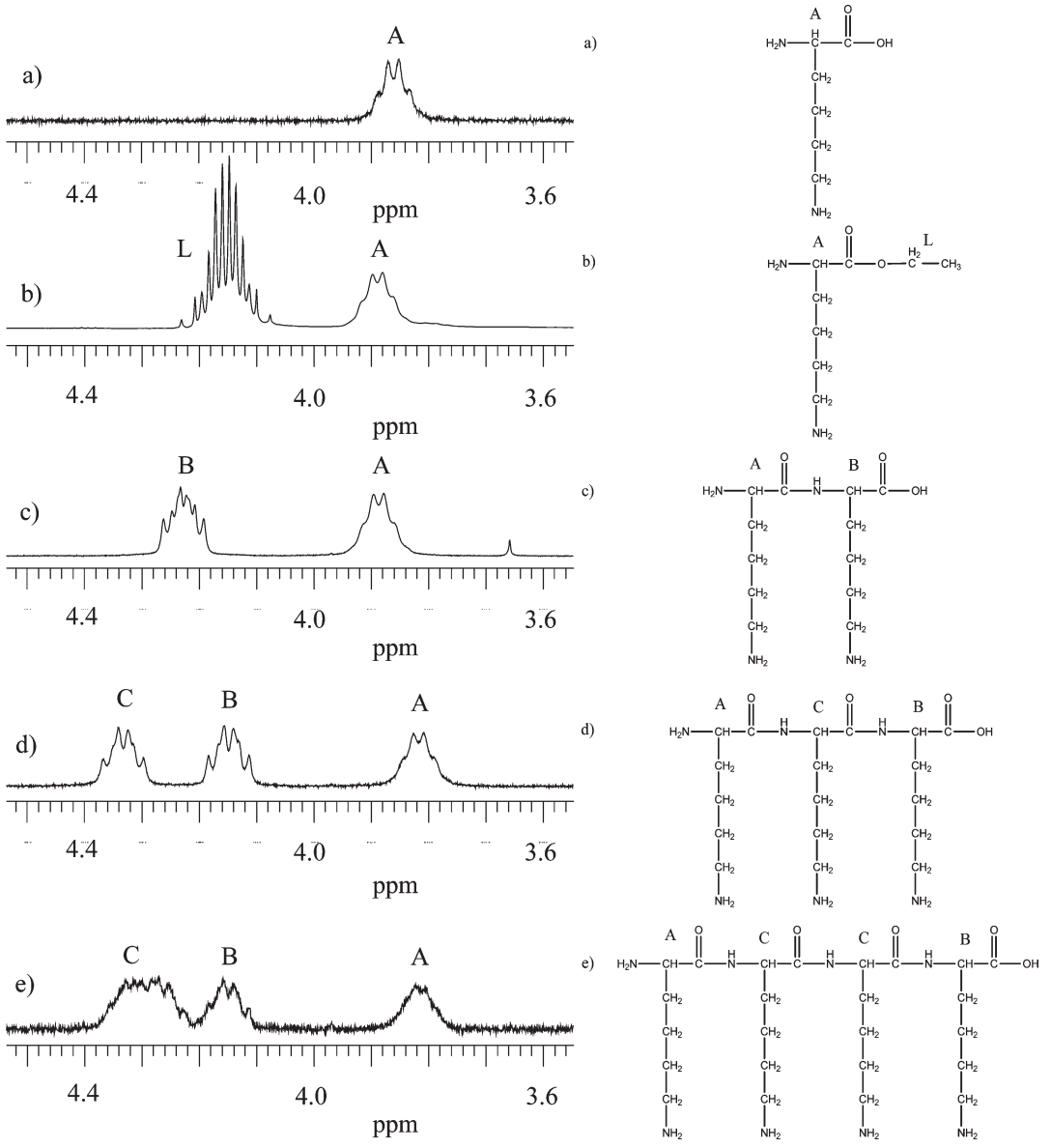

Figure 2. ^{1}H NMR (300 MHz, DMSO-d_6) spectra of L-Lys (a); L-Lys-Et (b); and model compounds oligo(L-Lys)$_2$ (c), oligo(L-Lys)$_3$ (d), and oligo-(L-Lys)$_4$ (e).

groups is proportional to the amount of the same Lys residuals. Figure 3 shows a product mixture from oligomerization of L-Lys-Et, including L-Lys-Et (residual monomer), L-Lys (hydrolyzed byproduct) and oligo(L-Lys) with various chain lengths. By comparing with coresponding peaks in Figure 2 for model compounds, peak A between 3.80 and 3.95 ppm in Figure 3 consists of overlapped methine peaks due to free L-Lys-Et and L-Lys in addition to N-terminal Lys units of oligo-(L-Lys). In addition, peak B·L between 4.12 and 4.23 consists of methine peaks B of C-terminal Lys units of oligo(L-Lys) and methylene protons L of L-Lys-Et ethyl groups. Since oligo(L-Lys) contains an equal quantity of N- and C-terminal L-Lys units, it follows that by subtracting the integration value of peak B from peak A (methine protons of N-terminal Lys units + free L-Lys-Et and L-Lys), the quantity of nonoligomerized L-Lys and L-Lys-Et is obtained. Hence, percent monomer conversion to oligo(L-Lys) is determined by the following relationship using experimentally determined peak integration values:

$$\text{percent monomer conversion} = [1 - (A - B)/(A + B + C)] \times 100 \quad (1)$$

Since the number of N- or C-terminal Lys units is equal to the number of oligo(L-Lys) chains, DP_{avg} values of oligo(L-Lys) is determined by the following relationship using experimentally determined peak integration values:

$$DP_{avg} = (2B + C)/B \quad (2)$$

Given that B = B·L − L, L = 2/3M, M = K·M − K, and K = 3/2J, the following relationship was obtained:

$$B = B{\cdot}L - [2/3(K{\cdot}M - 3/2J)] \quad (3)$$

By taking the expression of *B* given in eq 3 and substituting this into eqs 1 and 2, equations to determine percent monomer

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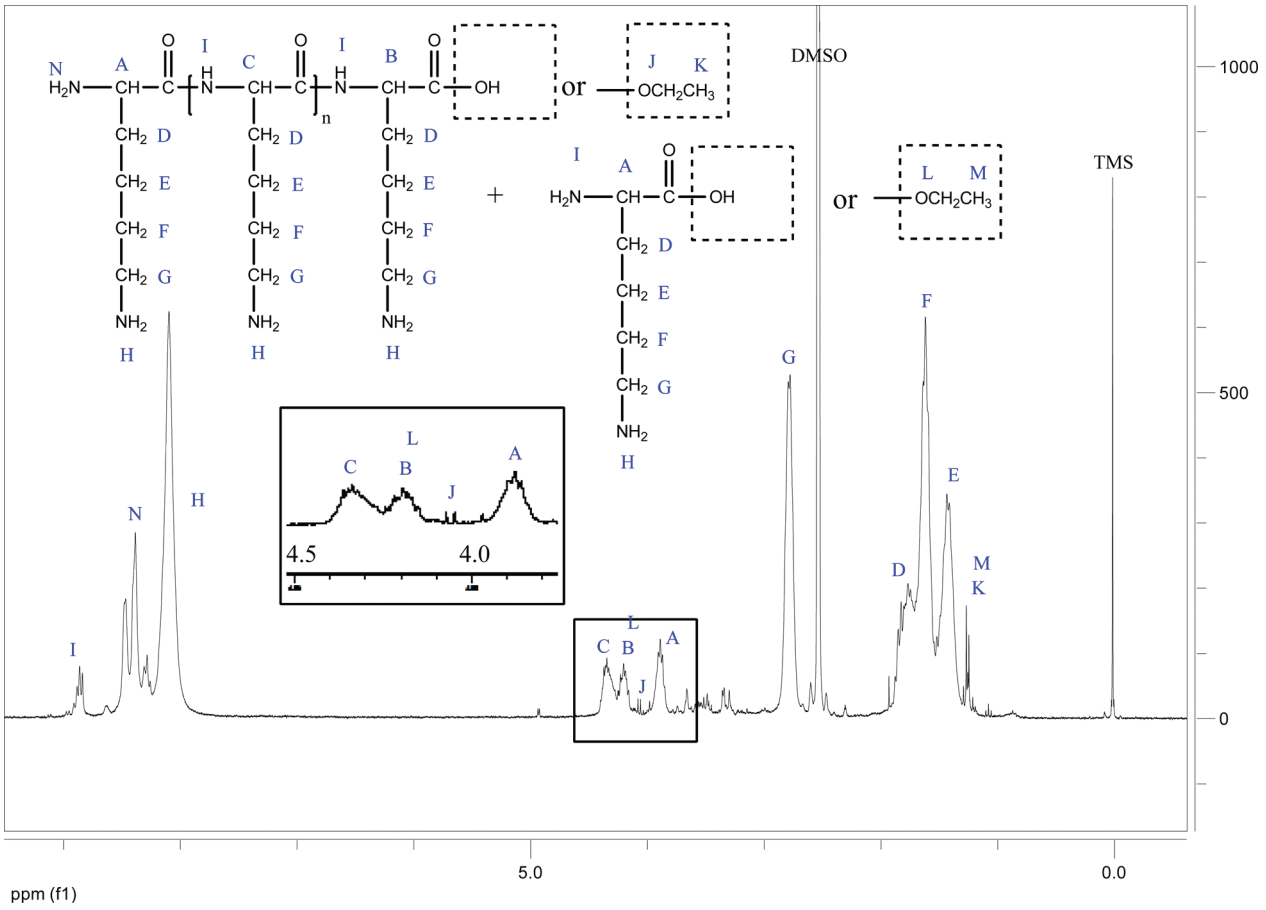

Figure 3. ^{1}H NMR (300 MHz, DMSO-d_6) spectra of oligo(L-Lys) prepared by bromelain (20 mg/mL) catalysis from 0.5 M L-Lys-Et at pH 7.6 (pH stat controlled), 40 °C and for 1.5 h.

conversion to oligo(L-Lys) and oligo(L-Lys) DP_{avg} from readily measured oligo(L-Lys) peak integrations were developed.

$$\text{percent monomer conversion} = \{1 - \{A - [B\cdot L - 2/3(K\cdot M - 3/2J)]\} / \{A + [B\cdot L - 2/3(K\cdot M - 3/2J)] + C\}\} \times 100 \quad (4)$$

$$DP_{avg} = \{2[B\cdot L - 2/3(K\cdot M - 3/2J)] + C\} / [B\cdot L - 2/3(K\cdot M - 3/2J)] \quad (5)$$

In most cases, the rapid hydrolysis of C-terminal ethyl ester bonds results in signals corresponding to protons J having a negligible contribution so that eqs 4 and 5 can be simplified as follows:

$$\text{percent monomer conversion} = \{1 - [A - (B\cdot L - 2/3K\cdot M)] / [A + (B\cdot L - 2/3K\cdot M) + C]\} \times 100 \quad (6)$$

$$DP_{avg} = [2(B\cdot L - 2/3K\cdot M) + C]/[B\cdot L - 2/3K\cdot M] \quad (7)$$

When monomer consumption is moderate (50−95%), percent monomer conversion and DP_{avg} values are determined by eqs 6 and 7. When monomer consumption is high (>95%), since peaks L and M are small compared with peaks B and K, respectively, peak B·L ≈ B, and K·M is neglectable. Hence, eqs 4 and 5 can further be simplified to eqs 1 and 2, respectively. When monomer consumption is low (≤50%), residual L-Lys-Et in reaction mixtures is determined using the integration intensity of methyl protons K (Figure 3). Although the signal of protons M is not resolved from signal K corresponding to the methyl groups of L-$(Lys)_n$-Et in peak K·M, the intensity of methyl groups K of L-$(Lys)_n$-Et can be neglected for percent monomer conversion values below 50. Therefore, K·M ≈ M, and DP_{avg} values of oligo(L-Lys) are determined by the following relationship using experimentally determined peak integration values:

$$DP_{avg}\ \text{values} = [(B\cdot L - 2/3M) + (B\cdot L - 2/3M) + C] / (B\cdot L - 2/3M) \quad (8)$$

PROTEASE SCREENING AT DIFFERENT PHS FOR OLIGOMERIZATION OF L-LYSINE ETHYL ESTER

Prior to determining the effect of different parameters on the synthesis of oligo(L-Lys), experiments were first performed to select a protease with the highest catalytic efficiency. Previous studies on protease catalysis demonstrated the optimal pH to hydrolyze and synthesize peptide bonds can differ substantially.[40] Since many types of proteases can potentially be used for in vitro protease-catalyzed transformations, four proteases were selected for initial evaluation on the basis of the following information. Aso et al.[32] have already shown that trypsin is active for oligo-(L-Lys) synthesis. Furthermore, data compiled in the literature on protease-catalyzed hydrolysis shows that, for papain, bromelain, and trypsin, the preferred cleavage site (-↓-) between peptide

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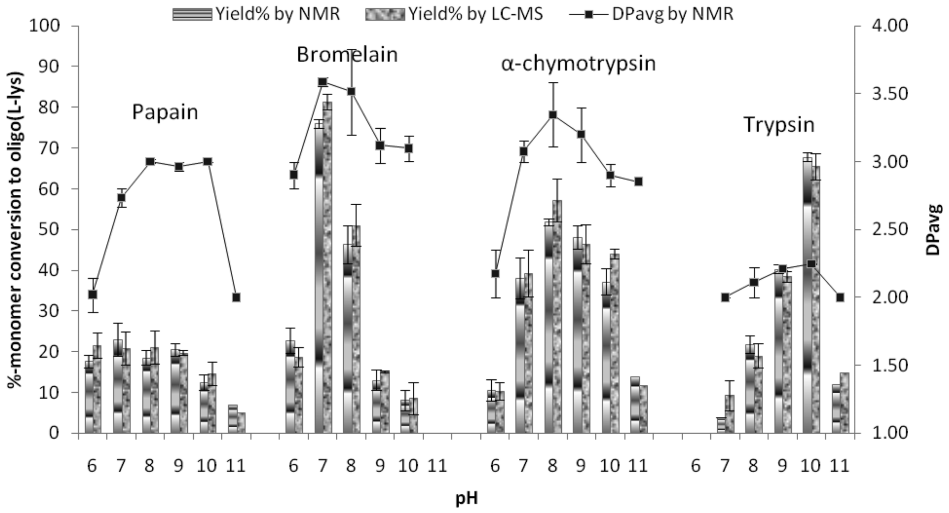

Figure 4. Percent monomer conversion and DP_{avg} values for oligo(L-Lys) synthesis catalyzed by papain, bromelain, α-chymotrypsin and trypsin as a function of pH. Reactions were conducted with 0.5 M L-Et-Lys, 16 units/mL catalyst, 40 °C for 1.5 h. Error bars represent the deviation from the mean of duplicate experiments.

positions P_1 and $P_{1'}$ is −Lys-↓-Z, where Z represents amino acid residues, esters, or amides.[41,42] Furthermore, previous work by our laboratory and others has shown that the proteases papain, bromelain, α-chymotrypsin, and trypsin are good catalysts for oligopeptide synthesis from a variety of amino acid ethyl ester monomers.[24,25,43] Hence, on the basis of the above, papain, bromelain, α-chymotrypsin, and trypsin were selected for initial evaluations of their relative percent monomer conversion (percent yield) and DP_{avg} values for oligo(L-Lys) synthesis from Et-L-Lys. Following the method of Li et al.,[25] the quantity of protease added to reactions was normalized on the basis of their relative activities for casein hydrolysis in which a unit is the quantity of protease required to release 1 μmol of tyrosine equiv min^{-1} mL^{-1} in buffer at 37 °C. It is well-known that protease activity for oligopeptide synthesis is pH-dependent. Furthermore, pH dependence may be further complicated by variation in substrate cationic character with pH. Hence, evaluations of the relative percent monomer conversion and DP_{avg} values for oligo(L-Lys) synthesis catalyzed by papain, bromelain, α-chymotrypsin, and trypsin was evaluated as a function of pH using a pH stat for pH control, and the results are plotted in Figure 4.

Inspection of percent monomer conversion values determined by ^{1}H NMR and LC−MS given in Figure 4 shows they are in excellent agreement, supporting the validity of both methods. 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. These results are consistent with compiled literature data that shows Lys-↓-Z is a preferred cleavage site for trypsin and bromelain, with the assumption that such reactions are reversible.[41,42] An additional reference providing preferred cleavage sites of proteases has been compiled by Sigma-Aldrich.[44] For the convenience of readers, the information from these web resources is available in the Supporting Information (Table S1).

Although papain prefers similar cleavage sites where Lys occupies the P_1 position, papain also prefers hydrophobic amino acids to occupy the P_2 position (Table S1 of the Supporting Information[44]). Examples of preferred amino acids at P_2 are Phe, Val, and Leu. This preference at P_2 is consistent with the observed low L-Lys percent conversion values by papain (up to 23 ± 4% at pH 7). Although papain prefers hydrophobic amino acids at P_2, α-chymotrypsin preferred hydropohobic amino acids such as Trp, Tyr, and Phe at the P_1 site. In contrast to papain, percent monomer conversions up to 52 ± 1% at pH 8 were obtained by α-chymotrypsin catalysis, showing that, in this case, preferred cleavage sites predicted by Schecter and Berger[42] did not foretell the outcome. However, as given in Table S1,[44] α-chymotrypsin shows broad selectivity on preferential cleavage sites between any two amino acids except Pro, Glu, Asp, and Gly at P1 and Pro at P1′. Thus, on the basis of Table S1,[44] successful oligomerization of L-Lys by α-chymotrypsin such that percent monomer conversions of more than 40% were obtained over a wide pH range (7 to 10) is within the boundary conditions of predicted enzyme selectivity.

Sensitivity of percent monomer conversion values to medium pH was highly variable for the four proteases studied. Bromelain and trypsin showed the highest sensitivity to medium pH. For bromelain, percent monomer conversion varied from 23 ± 1% to 76 ± 2% and 46 ± 5% for pH values of 6, 7, and 8, respectively. Similarly, using trypsin as catalyst, percent monomer conversion varied from 40 ± 1% to 68 ± 1% and 12% for pH values of 9, 10, and 11, respectively. This agrees well with Aso et al.,[32] who also found the highest L-Lys-Et consumption by trypsin-catalyzed oligomerization at pH 10. In contrast, papain catalysis gave percent monomer conversion values that showed small variation as a function of medium pH. In addition, α-chymotrypsin showed a broad pH range at which percent monomer conversion varied little.

It might be presumed that there would be good correlation between the sensitivity to medium pH of values for percent monomer conversion and DP_{avg} for a given protease. However, with the exception of papain, this is not the case for the protease−(L-Lys-Et) systems studied herein. This is best illustrated for bromelain and trypsin, both of which showed high variability of percent monomer conversion values with changes in medium pH. However, for trypsin, DP_{avg} varied little (2−2.3) from pH 7 to 11. For bromelain, DP_{avg} varied between 8 ± 3% and 76 ± 1% from pH 6 to 10. This leads to the important

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conclusion that trends for percent monomer conversion and DP_{avg} do not necessarily correlate. This is true when considering the effects of pH for a given protease as well as when comparing the efficiencies of different proteases for oligo(L-Lys) synthesis. For instance, when considering only DP_{avg}, differences between papain, bromelain, and α-chymotrpysin are relatively small. Another important finding from the results in Figure 4 is that of the four proteases, trypsin gives the lowest values of DP_{avg}. Since bromelain gives the highest value of both DP_{avg} (3.6) and percent monomer conversion (76 ± 1%), bromelain was selected for more detailed studies described below that elucidate how other reaction parameters influence oligo(L-Lys) synthesis.

EFFECT OF PH VALUE ON BROMELAIN-CATALYZED OLIGO(L-LYS) SYNTHESIS

Figure 5a and b maps the effects of reaction pH on bromelain-catalyzed oligo(L-Lys) synthesis for values from 6 to 9 and pH intervals ≤0.4 units. At pHs from 7 to 8, where percent monomer conversion and DP_{avg} values were highest (Figure 4), data was obtained at 0.1 pH intervals. All experiments were performed in water with precise pH control by pH stat. Indeed, within the pH range from 7 to 7.8, percent monomer conversion was ≥75%, and DP_{avg} ≥ 3.5. Maximum values of percent monomer conversion and DP_{avg} were 84 ± 2% and 4.1 at pH values 7.6 and 7.8, respectively. In contrast, optimal pH values for bromelain-catalyzed peptide hydrolysis reactions is from 4.5 to 5.5,[25] about 2.5 units lower than preferred pH values for peptide synthesis. The preference for higher pH values during peptide synthesis reactions has been noted before by us and others.[24,32] Observation of the pH region from 7.8 to 8.2 shows that, with an increase in pH of only 0.4 units, the percent monomer conversion decreases from 82 ± 2% to 23 ± 5% (Figure 5a), and DP_{avg} decreases from above 4 to below 3.3 (Figure 5b). Similarly, on the low pH side of optimal values, a decrease in the pH from 6.7 to 6.4 resulted in a decrease in percent monomer conversion from 74 ± 3% to 35 ± 3%, and a decrease in reaction pH from 7.0 to 6.4 resulted in a decrease in DP_{avg} from 3.6 ± 0.0 to 2.8 ± 0.0.

To gain insight into how reaction pH influences the occurrence of hydrolysis reactions (e.g., L-Lys-Et to L-Lys), the accumulation of L-Lys in reactions was measured by LC−MS (see Instrumental methods section). At pH values above 8, an increase in L-Lys in oligomerizations was determined by subtracting the amount of L-Lys in the control reactions (no enzyme) from the experimental values. Within the pH range from 7 to 7.6, where percent monomer conversion and DP_{avg} have relatively high values, the mole percent of L-Lys remains low (i.e., between 14 and 18%). However, in the pH range between 7.6 and 8.0, where large decreases in percent monomer conversion and DP_{avg} occur, there is a corresponding increase in mole percent of L-Lys, from ~16 to 30%, and above pH 8, bromelain activity for peptide bond synthesis and ester hydrolysis is low. A time course study that begins to address the sequence of events by which different species are formed and consumed during bromelain catalyzed oligo(L-Lys) synthesis is given below.

EFFECT OF MONOMER AND ENZYME CONCENTRATION

Previous studies on the influence of monomer and protease concentration have focused on papain-catalyzed oligo(γ-Et-L-glu) and bromelain-catalyzed oligo(L-phe).[14,24] Unlike the study herein, in which oligo(L-Lys) remains in the aqueous phase

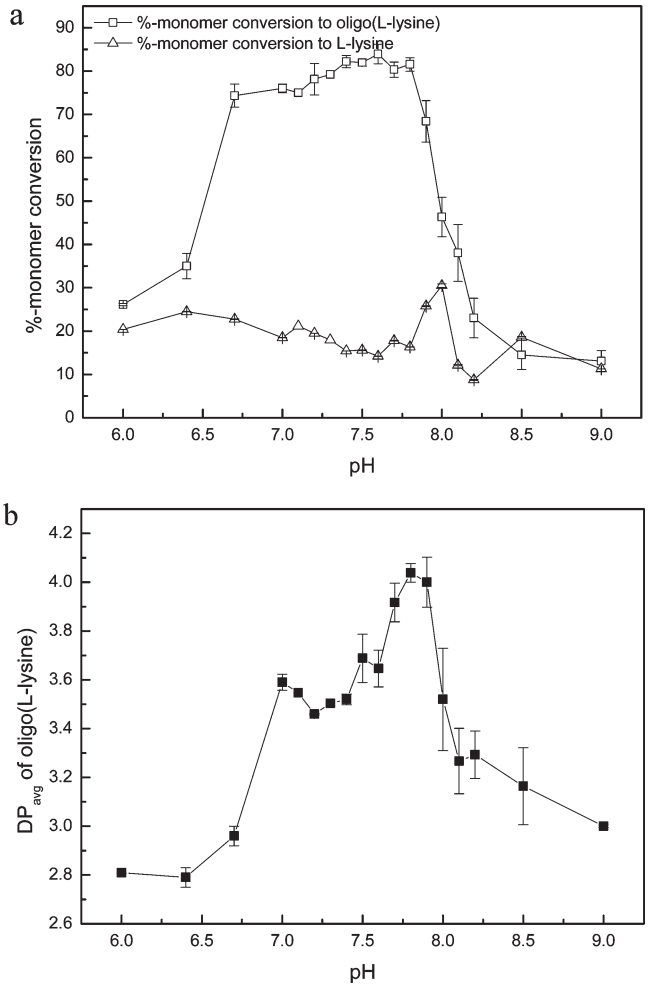

Figure 5. Effects of reaction pH on (a) percent monomer conversion to oligo(L-Lys) with generation of L-Lys and (b) DP_{avg}. Oligomerizations were conducted with 0.5 M monomer, 16 units/mL bromelain, at 40 °C, for 1.5 h. Error bars represent the deviation from the mean of duplicate experiments.

during synthesis, oligo(γ-Et-L-glu) and oligo(L-phe) precipitate from the reaction media as they approach specific chain lengths. Precipitation drives the equilibrium toward peptide formation, and the resulting insoluble products, as a consequence of being in a heterogeneous phase relative to the protease, have greatly reduced susceptibility to protease-catalyzed hydrolysis to monomer or lower oligomer chain lengths (see Scheme 1c).

Figure 6a displays the effect of L-Lys-Et concentration on percent monomer conversion to oligo(L-Lys) and DP_{avg}. For this set of experiments, the bromelain concentration was fixed at 20 mg/mL and the pH was held at 7.6, a value preferred for achieving both high oligo(L-Lys) yield and DP_{avg} values. By increasing the monomer concentration from 0.25 to 0.75 M, the percent monomer conversion changed little, whereas DP_{avg} increased from 3.1 ± 0.0 to 3.9 ± 0.1. A further increase in monomer concentration from 0.75 to 1.5 M results in a decrease in the percent monomer conversion from 83 ± 2% to 21 ± 8% and oligo(L-Lys) DP_{avg} from 3.9 ± 0.1 to 3.0 ± 0.0. Within the monomer concentration range from 0.25 to 0.75 M, where percent monomer conversion and DP_{avg} have relatively high values, the mole percent of L-Lys remains low (i.e., between 11 ± 1% and

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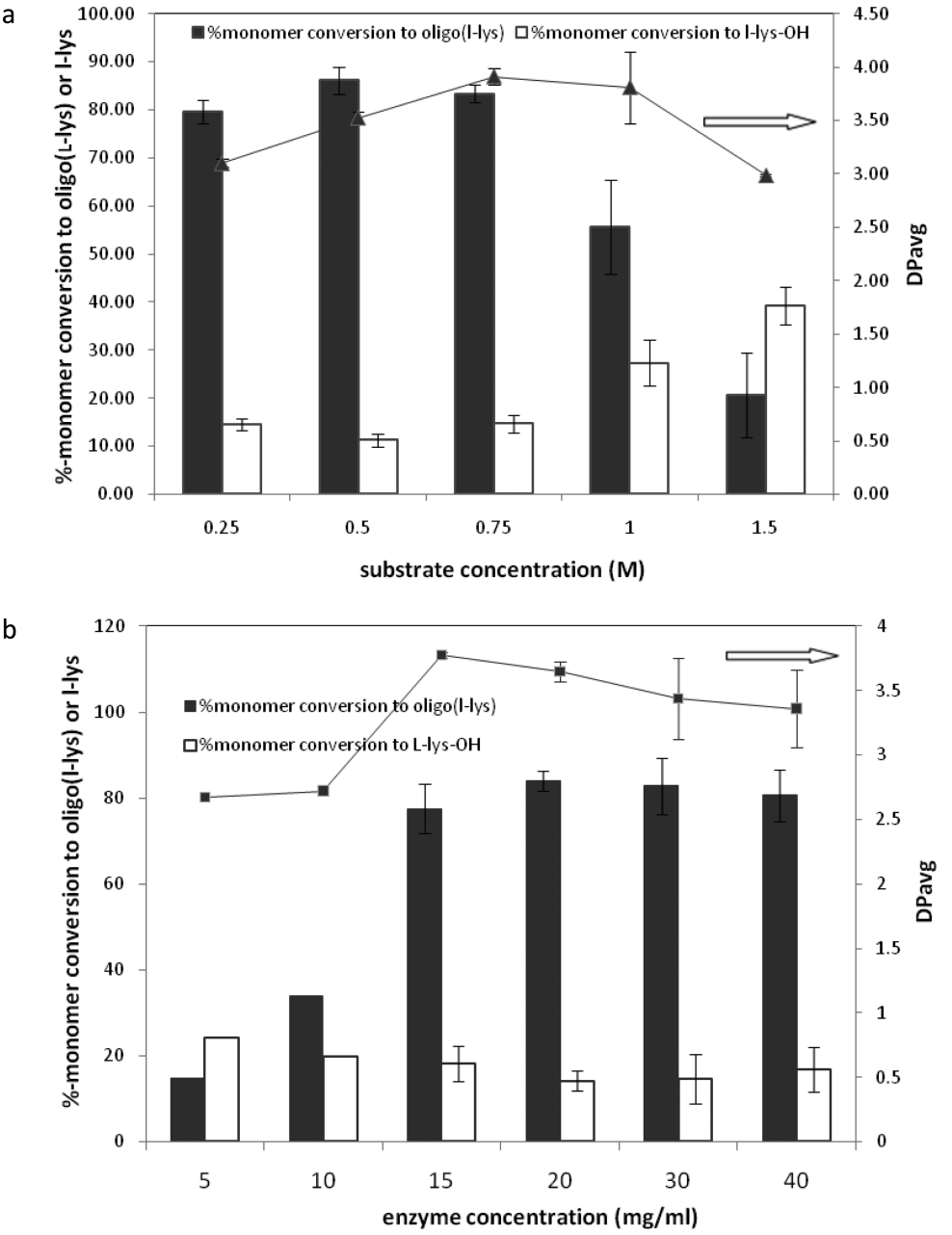

Figure 6. Effects on percent monomer conversion to oligo(L-Lys) and DP_{avg} of variation in (a) monomer (L-Lys-Et) concentration with bromelain concentration fixed at 20 mg/mL and (b) bromelain concentration with monomer concentration fixed at 0.5 M. Reactions were conducted at 40 °C for 1.5 h. Error bars represent the deviation from the mean of duplicate experiments.

15 ± 2%). However, as the L-Lys-Et concentration is increased to 1 and 1.5 M, where large decreases in percent monomer conversion and DP_{avg} occur, there is a corresponding increase in mole percent of L-Lys to 27 ± 5% and 39 ± 4%, respectively. Thus, as was observed in Figure 5a, where pH was increased to 7.8 and above, as bromelain activity for peptide bond synthesis decreased with increased monomer concentration, a corresponding increase in hydrolysis activity occurred. Furthermore, on the basis of the proposed mechanism in Scheme 1, an increase in the monomer concentration with fixed enzyme concentration will lead to increased concentrations of ES^{Lys}, formation of higher concentrations of NH_2−Lys-Lys−OEt, and longer oligomers.

Since monomer concentrations below 0.25 M were not studied, this evolution in percent monomer conversion and DP_{avg} values with increased substrate concentration was not observed. However, this phenomena was documented in previous publications on papain-catalyzed synthesis of oligo(γ-Et-L-glu) and bromelain-catalyzed synthesis of oligo(L-phe).[14,24] In addition, a study below where the monomer concentration is fixed while the enzyme concentration is varied provides insight into the enzyme−substrate concentration region where percent monomer conversion and DP_{avg} values increase. Thus, in the L-Lys-Et concentration region of 0.25 to 0.75M, it appears that substrate saturated conditions are attained. It then follows that at 1 and 1.5 M monomer concentrations, within the substrate saturated

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concentration regime, total monomer conversion with time will remain constant, and therefore, percent monomer conversion as a function of time will decrease, leading to accumulation of L-Lys in reaction media, as was observed in Figure 6a. Furthermore, at substrate saturation, [ES^{Lys}] remains constant as [L-Lys-Et] increases, leading to an increase in the number of chains formed and relatively shorter oligomer species dominate the product population.

Figure 6b illustrates how percent monomer conversion to oligo(L-Lys) and DP_{avg} are affected by variation in bromelain concentration with pH held at 7.6 and monomer concentration fixed at 0.5M. An increase in bromelain concentration from 5 to 15 mg/mL resulted in increases in percent monomer conversion and oligomer DP_{avg} from 15 to 78 ± 6% and 2.7 to 3.8 ± 0.0, respectively. However, as the bromelain concentration was increased from 15 to 40 mg/mL, the percent monomer conversion and oligomer DP_{avg} remained nearly unchanged. An increase in the catalyst concentration with constant monomer concentration should lead to an increase in the rate of ES^{Lys} formation and oligomer synthesis. This is, indeed, observed for bromelain concentrations between 5 and 15 mg/mL. A further increase in the catalyst concentration should further increase the rate of ES^{Lys} formation and oligomer synthesis. However, on the basis of observing products at 1.5 h, this was not found. These results indicate that, for a monomer concentration of 0.5 M and bromelain concentrations between 20 and 40 mg/mL, reactions are complete in ≤1.5 h. Furthermore, the product formed must be relatively stable so that the percent monomer conversion to oligomer and DP_{avg} remain invariable.

EFFECT OF INCUBATION TEMPERATURE

On the basis of previous reports by our research group that established 40 °C as the preferred temperature for bromelain-catalyzed oligomerization of L-phe-Et,[14] 40 °C was similarly adopted for the above studies on L-Lys-Et oligomerization. However, it is well established that the optimal temperature of an enzyme may vary as a function of the specific reaction system under study. Hence, a study of the influence of the reaction temperature (10, 25, 40, 55, and 70 °C) on percent monomer conversion to oligo(L-Lys) and oligo(L-Lys) DP_{avg} was conducted, and the results are displayed in Figure 7. Maximum percent monomer conversion values of >80% were obtained by carrying out reactions at 25 and 40 °C. Even at 10 °C, the percent monomer conversion remained high (74 ± 4%). At temperatures from 10 to 40 °C, oligo(L-Lys) DP_{avg} remained at about 3.6, and the mole percent of L-Lys accumulated in reactions due to ester hydrolysis was between 10 and 15% (latter not shown in Figure 7). Indeed, in studies above, regardless of the parameter varied, when the percent monomer conversions reached between 75 and 85%, DP_{avg} values were generally between 3.5 and 4.0, and the mole percent of L-Lys accumulated in the reactions was between 10 and 20%.

TIME COURSE OF OLIGO-L-LYSINE SYNTHESIS

On the basis of the kinetic model of amidation by cysteine and serine proteases, to obtain the optimal oligo(L-Lys) percent yield and DP_{avg} values, an understanding of the time course of product formation and subsequent hydrolysis reactions is needed. In other words, since the thermodynamic product of reactions performed herein is L-Lys, it is necessary to determine the reaction time that corresponds to the formation of oligo(L-Lys)

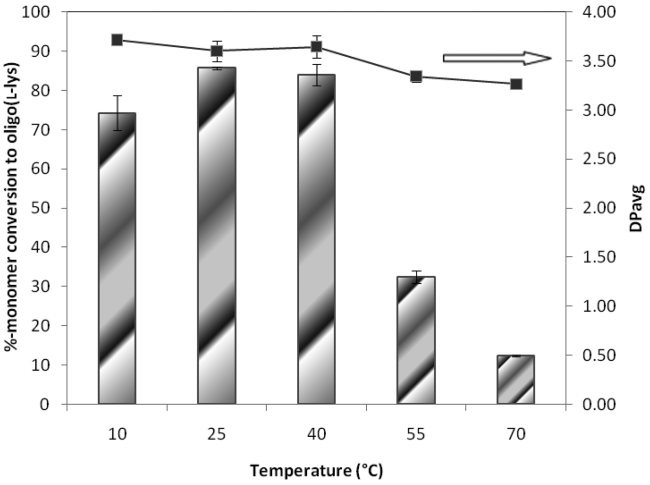

Figure 7. Effects of temperature on percent monomer conversion to oligo(L-Lys) and oligo(L-Lys) DP_{avg}. Reactions were conducted in deionized water at pH 7.6 with 20 mg/mL bromelain and 0.5 M L-Lys-Et for 1.5 h. Error bars represent the deviation from the mean of duplicate experiments.

in the highest yield and DP_{avg} with the desired distribution of oligomer chain lengths. For this study, the reaction was performed by selecting the optimal reaction parameters determined above (i.e., pH 7.6, 40 °C, 20 mg/mL bromelain, and 0.5 M L-Lys-Et).

Figure 8a shows that, by 15 and 30 min, the percent monomer conversion to oligo(L-Lys) rapidly increased to 69 ± 8 and 85 ± 4%, respectively. The percent monomer conversion to oligo(L-Lys) remains at ~80%, even though the reaction time is 3 h, which is twice as long as the experiments discussed above. In addition, formation of L-Lys by hydrolysis reactions was rapid so that, by 15 and 30 min, L-Lys reached 5 ± 0 and 11 ± 3 mol %, respectively, of the initial L-Lys-Et concentration. As the reaction continued to 3 h, L-Lys reached 15 mol % by 40 min and did not exceed that value thereafter. Residual L-Lys-Et was only 2% by 40 min. Furthermore, Figure 8b shows that by 30 min, DP_{avg} and the longest oligomer chain length observed by LC−MS reached maximum values of ~3.6 and 12.0, respectively. As the reaction time progressed to 3 h, DP_{avg} did not substantially change, but the longest observed oligomer chain length decreased to 9 by 1.5 h. Hence, these results show that bromelain-catalyzed conversion of L-Lys-Et to oligo(L-Lys) occurs rapidly so that by 30 min, maximum values of percent monomer conversion to oligo(L-Lys) and DP_{avg} and the longest observed oligo(L-Lys) chain length were reached.

On the basis of the above discussion, oligo(L-Lys) synthesis was complete by 30 min and, with the exception of oligo(L-Lys) chains with DP 10, 11, and 12, which were degraded presumably by protease hydrolysis (Scheme 1c), products formed by 30 min remained relatively unchanged for the following 2.5 h. This can be explained by the rate of bromelain-catalyzed hydrolysis of oligo(L-Lys) being slow. A contributing factor to this behavior may be decreased bromelain activity due to self-digestion. These results are consistent with the results shown in Figure 6b, where it was concluded that (a) since an increase in the catalyst concentration did not result in further increases in oligomer synthesis, then the reactions are complete in ≤1.5 h, and (b) the product formed must be relatively stable so that percent monomer

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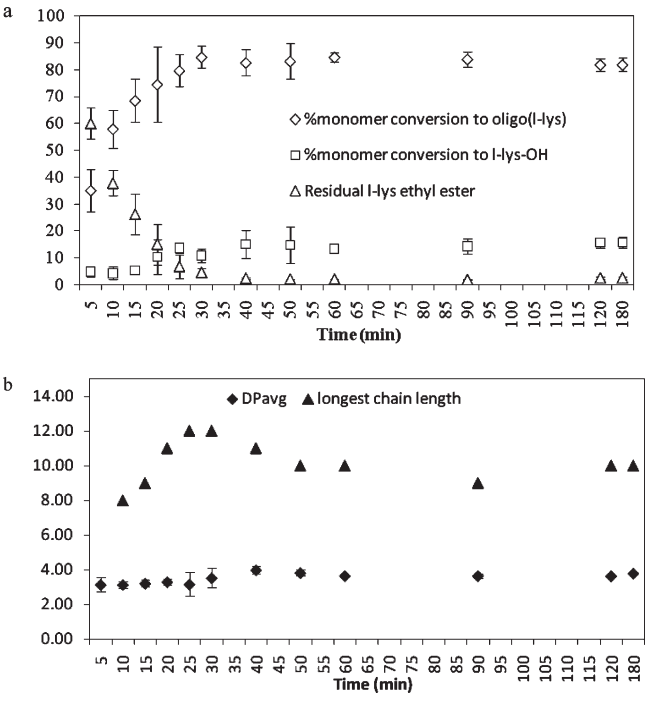

Figure 8. Time course study of bromelain-catalyzed synthesis of oligo(L-Lys). The reaction was conducted at pH 7.6, 40 °C, with 20 mg/mL bromelain and 0.5 M L-Lys-Et. Results in panel a give values of oligo(L-Lys) percent yield, formation of L-Lys-OH, and consumption of L-Lys-Et; results in panel b give oligo(L-Lys) DP_{avg} and the longest oligo(L-Lys) chain length observed by LC−MS.

conversion to oligomer and DP_{avg} remained invariable with the increased catalyst concentration.

SUMMARY OF RESULTS

A mechanism was presented that describes the sequence of reactions believed to occur during conversion of L-Lys-Et to oligo(L-Lys). The mechanism depicts key steps during chain initiation and propagation as well as describing competitive pathways that can lead to monomer deactivation and chain hydrolysis. In addition, the mechanism calls attention to alternate pathways by which chain propagation may occur, that is, one amino acid at a time from chain ends versus by condensation-type step reactions between oligomers. Water can also act as a competitive acyl acceptor to form hydrolyzed free L-Lys.

Through a comparative analysis of ^{1}H NMR spectra of L-Lys, L-Lys-Et and oligomer standards oligo(L-Lys)$_2$, oligo(L-Lys)$_3$ and oligo(L-Lys)$_4$, assignments were made of internal and terminal methine signals of oligo(L-Lys) mixtures synthesized by protease catalysis. This information was used to determine DP_{avg} and percent monomer conversion values of reactions performed herein.

To analyze the chain length distribution of synthesized oligo(L-Lys) products as well as to obtain an independent value of DP_{avg}, heptafluorobutyric acid (HFBA) was used in the mobile phase to ion-pair oligo(L-Lys) mixture components to increase its interactions with a C18 reversed-phase column. This resulted in baseline resolution of constituent oligomers in mixtures and identification of their chain lengths by mass spectrometry. The use of UV detection was found to give DP_{avg} values of oligolysine mixtures that were in excellent agreement with those determined by ^{1}H NMR.

Papain, bromelain, α-chymotrypsin, and trypsin were selected for evaluations of their relative activities for conversion of Et-L-Lys to oligo(L-Lys). Results obtained were compared with that predicted by data compilations of preferred cleavage sites (-↓-). 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%) by maintaining the reaction pH between 7.6 and 7.8.

Bromelain-catalyzed L-Lys-Et oligomerization reactions can be successfully performed over a wide range of temperatures. Remarkably, oligomerizations conducted at 10 °C for 1.5 h gave high percent monomer conversions (74 ± 4%). Furthermore, at temperatures from 10 to 40 °C, oligo(L-Lys) DP_{avg} remained at ∼3.6.

Since oligo(L-Lys) remains soluble in the reaction media, the possibility exists that beyond an optimal point in time, products formed might be degraded. This prompted a time course study. By adopting preferred reaction parameters (i.e., pH 7.6, 40 °C, 20 mg/mL bromelain, and 0.5 M L-Lys-Et) it was discovered that oligomers with chain lengths of more than 10 are formed within 5 min. The DP_{avg} and longest oligomer chain lengths reached maximum values of ∼3.6 and 12.0, respectively, by 30 min. Surprisingly, extending the reaction to 3 h did not significantly change the DP_{avg}, percent monomer conversion, or free L-Lys in the reaction medium.

Further work is in progress to determine kinetic parameters of competing reactions that will provide greater insights on the reaction mechanism. A focus of future work by our laboratory will be to identify methods by which competitive hydrolysis reactions can be minimized and longer chain length oligomers can be prepared.

ASSOCIATED CONTENT

Supporting Information. COSY-NMR (300 MHz, DMSO-d_6) spectra of oligo(L-Lys) and preferred cleavage sites of papain, bromelain, α-chymotrypsin, and trypsin. This material is available free of charge via the Internet at http://pubs.acs.org.

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