

Protease-Catalyzed Oligomerization of Hydrophobic Amino Acid Ethyl Esters in Homogeneous Reaction Media Using L-Phenylalanine as a Model System

Kodandaraman Viswanathan, Ruth Omorebokhae, Geng Li, and Richard A. Gross*

Department of Chemical and Biological Science, NSF I/UCRC for Biocatalysis and Bioprocessing of Macromolecules, Polytechnic Institute of NYU, Six Metrotech Center, Brooklyn, New York 11201

Received May 11, 2010; Revised Manuscript Received June 24, 2010

Enzymatic synthesis of oligopeptides from L-phenylalanine ethyl ester hydrochloride (L-Phe-Et·HCl) and other L-form hydrophobic amino acid ester hydrochlorides in water miscible organic cosolvents was studied. Different proteases, water miscible cosolvents, and effect of different ratios of water miscible cosolvents for protease-catalyzed oligo-phenylalanine [oligo(L-Phe)] were compared. The importance of the use of water miscible cosolvents in transforming reactions from heterogeneous to homogeneous conditions as a potent medium engineering tool for protease-catalyzed oligopeptide synthesis is highlighted. For example, at 0.125 M L-Phe-Et·HCl, 20% (v/v) methanol, 18.6 mg/mL bromelain, in phosphate buffer (0.25M, pH 8), 40 °C, for 3 h, oligo(L-Phe) precipitated from the solution to yield $45 \pm 5\%$, in contrast, in the absence of cosolvent oligo(L-Phe) yield of $29 \pm 5\%$ was obtained. The following reaction conditions were optimized for bromelain catalyzed oligo(L-Phe) synthesis: pH, temperature, substrate, enzyme, and cosolvent concentrations. DP_{avg} and chain length distribution in the product peptides were investigated by ^1H NMR and MALDI-TOF.

Introduction

Peptides are molecules of paramount importance in the field of health care and nutrition. Furthermore, inclusion of bioactive peptides in various biomaterials is a key strategy to achieve desired biological properties such as for drug targeting or to modulate cell differentiation activities.^{1–3} Also, peptides are important components of soft materials prepared by self-assembly. For example, dipeptides of phenylalanine organize within the central aromatic core of β -amyloid polypeptides that self-assemble into hollow nanotubes.⁴ Staining with ionic silver followed by proteolytic digestion of phenylalanine dipeptide produced nanowires of ~ 20 nm in diameter.⁴ Short peptides synthesized by solid phase peptide synthesis with 8-mer units that consist of 1–2 aspartic acid units with hydrophobic amino acids such as alanine, valine, or leucine, self-assemble into nanotubes and nanovesicles.⁵ Established approaches in synthetic peptide chemistry are routinely used to produce peptides and small proteins of biological and pharmaceutical importance.^{6–8} Precise sequence peptides synthesized by solid phase chemical methods have also been conjugated to hydrophobic and hydrophilic molecules such as lipids and polyethylene glycol (PEG) to attain molecules with self-assembling characteristics.^{3,9} These self-assembled amphiphilic peptide conjugates are being studied for drug and metal binding for a wide range of medical and pharmaceutical applications.^{3,9,10}

To prepare peptides with a precise sequence of amino acids, typically solid phase or liquid phase peptide synthesis is used.^{11,12} While these methods provide the desired peptides in high purity, they are costly due to the use of toxic reagents, protection–deprotection chemistry, and product purification. An alternative method to preparing sequence and chain length precise peptides is via protein engineering of desired sequences into organisms and production via fermentation. However,

fermentative peptide synthesis generally gives low product yields and requires specialized methods if non-natural amino acids are desired within chains.^{13,14} The high cost of these methods for peptide synthesis is limiting with regard to the expanding range of exciting applications for which peptides are contemplated. Therefore, new methods for peptide synthesis are needed that are safe, scalable, and cost-effective. Protease-catalyzed peptide synthesis is one such synthetic technique that circumvents the aforementioned difficulties associated with conventional synthetic approaches discussed above. Successes have been documented for protease-catalyzed dipeptide synthesis.^{15,16} However, protease-catalyzed synthesis of precise sequence peptides with >2 units remains challenging. Given the simplicity of protease-catalyzed peptide synthesis and the apparent ease by which it can be scaled-up, there is strong justification to further investigate the activity and specificity of known and recently discovered protease-catalysts on an array of natural and non-natural substrates for peptide synthesis. This paper is focused on the use of the serine and cysteine proteases, belonging to the family of endopeptidases, for oligomerization of hydrophobic amino acids that require the addition of a water-miscible cosolvent to attain homogeneous reaction media.

Publications by us and others have summarized important progress in protease-catalyzed routes to synthesize oligopeptides.^{17,18} The following summarizes research in which water miscible organic media such as dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), acetonitrile (ACN), and methanol (MeOH) were added at various concentrations to reaction media to solubilize poorly soluble hydrophobic substrates and curtail spontaneous hydrolysis of water-soluble products during protease-catalyzed oligopeptide synthetic transformations.¹⁹ Lobell et al.²⁰ reported coupling preformed oligopeptides in 10% acetonitrile borate buffer (pH 9) to prepare a calcium mimetic octapeptide.²⁰ Other reports include the coupling of Boc(D,L)Ala-OMe using papain in $\sim 40\%$ v/v MeOH and Boc(D,L)-Tyr-OEt using α -chymotrypsin in $\sim 40\%$ v/v DMF to H-Gly-NHNHPh,

* To whom correspondence should be addressed. E-mail: rgross@poly.edu.

respectively.²¹ The observed decrease in water activity by the addition of an inert organic solvent was ascribed to the decrease in water concentration.²² However, this result can also be explained by the fact that the addition of the organic cosolvent decreases protease hydrolytic activity.^{23,24} Andras et al.²⁵ investigated the extent of papain deactivation as a function of water miscible cosolvent structure and concentration by assaying casein hydrolytic activity. Addition of only 5–10% v/v THF to the aqueous medium resulted in large (38%) loss in activity. In contrast, addition of 90% v/v 1,4-dioxane, 90% v/v ethanol, and 90% v/v acetonitrile resulted in decreases in activity of 28, 25, and 64%, respectively.²⁵ These authors did not compare how addition of water-miscible solvents to aqueous papain solutions affects synthetic activity, which may not correlate to protein hydrolysis. Homandberg used 85% (v/v) 1,4-butanediol to increase K_{syn} for bovine α -chymotrypsin catalyzed coupling of glycineamide and Cbz-L-tryptophan to prepare Cbz-L-tryptophan-nylglycineamide.²⁶ Wong et al.²⁷ reported that amide bond formation between Z-L-Phe-OMe and L-Leu-NH₂ forming Z-Phe-Leu-NH₂, using α -chymotrypsin, papain, subtilisin, and protease type XXI from *Streptomyces griseus* as catalysts, was >10000 times faster in aqueous DMF (50% water, v/v) relative to in anhydrous DMF.²⁷ The use of water-miscible cosolvents can expand protease promiscuity, thereby enabling proteases to catalyze peptide synthesis with D-amino acid substrates.²⁸ Using α -chymotrypsin in free as well as immobilized form, West et al.²⁹ found that yields from reactions between Z-L-Tyr-OMe/Z-L-Phe-OMe and D-amino acid methyl esters (Met, Ser, Phe, Arg, Val, Leu, and Ala) increased for DMF and DMSO concentrations up to 50 and 60%, respectively. Furthermore, by increasing the DMF concentration to 50% for L-methionine ethyl ester hydrochloride oligomerization catalyzed by subtilisin BPN', increased oligo(L-methionine) chain lengths were observed so that, at 50% DMF, chains with average lengths of 50 units were formed.³⁰

Given the exciting potential of using protease catalysis to prepare a wide range of oligopeptides under simple reaction conditions, the extensive use of "medium engineering" to manipulate the relative rates of hydrolytic and amide forming reactions during dipeptide synthesis and coupling of preformed peptide segments and the promising initial findings by Wong et al.³⁰ when using water-miscible solvents to prepare oligo(L-methionine) with longer chain lengths, the authors determined that a need exists to further explore potential benefits and detriments of using water-miscible solvents to manipulate protease-catalyzed oligopeptide synthesis. Furthermore, when performing oligomerizations of L-Et-Phe·HCl using bromelain, papain, and α -chymotrypsin as catalysts at high pH and high buffer concentrations (0.25–0.9 M sodium phosphate) at 40 °C, it was observed at $t = 0$ that the L-Et-Phe·HCl phase separated. An immiscible system is undesirable because it complicates efforts to define reaction kinetics and to determine coreactivity of L-Et-Phe·HCl with other monomers that may or may not be water-soluble.

This paper explores the use of water-miscible solvents for amino acid ethyl ester solubilization and subsequent oligomerization by protease catalysis. Effect of cosolvents on papain-, bromelain-, and α -chymotrypsin-catalyzed oligomerization of L-Et-Phe·HCl was determined. From this information, bromelain and MeOH were selected as the preferred catalyst and cosolvent for systematic investigations of L-Et-Phe·HCl oligomerizations. A series of studies were performed in 20% MeOH phosphate buffer media to interrogate how the reaction pH, temperature, time, substrate concentration, and bromelain concentration

influences bromelain oligo(L-Phe) synthesis activity. These studies were then extended to determine the influence of MeOH cosolvent concentration on oligomerizations of L-Trp-Et, L-Et₂-Glu, L-Met-Et, and L-Leu-Et. Also, further studies were performed to determine affects of DMF and DMSO on bromelain hydrolytic and oligopeptide synthetic activity. The cumulative results of this work provide important insights into solubilization and oligomerization of a broad range of amino acid ethyl ester monomers using bromelain as catalyst.

Experimental Section

Materials. L-Phenylalanine ethyl ester hydrochloride [L-Et-Phe·HCl], L-leucine ethyl ester hydrochloride [L-Et-Leu·HCl], L-methionine ethyl ester hydrochloride [L-Et-Met·HCl], and L-tryptophan ethyl ester hydrochloride [L-Et-Tryp·HCl] were purchased from Sigma Aldrich, L-diethyl glutamic acid ethyl ester hydrochloride [L-(Et)₂-Glu·HCl] was purchased from Tokyo Kasei Co. Ltd., crude papain (cysteine protease EC 3.4.22.2; source, *Carcica papaya*; 30000 USP units/mg of solid; molecular weight 21K) was purchased from CalBiochem Co. Ltd., and bromelain (cysteine protease; EC 3.4.22.4; source pineapple stem; 3.4.22.32, protein content $\geq 35\%$ protein by biuret, 1.7 units/mg protein) was purchased from Sigma Aldrich. Water-insoluble materials in the as-received papain and bromelain were removed by following protocol in an earlier reference,¹⁷ α -chymotrypsin type II (serine protease; EC 3.4.21.1; source, bovine pancreas, 83.9 units/mg, 96 units/mg protein), sodium phosphate dibasic, sodium acetate, potassium chloride, casein, deuterated dimethyl sulfoxide (DMSO-*d*₆), deuterated trifluoro acetic acid (CF₃COOD), deuterated chloroform (CDCl₃), trifluoroacetic acid (CF₃COOH), Folin and Ciocalteu's reagent, trichloroacetic acid, and α -cyano hydroxycinnamic acid (CCA, MALDI-TOF matrix) were all purchased from Aldrich, acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), and tetrahydrofuran (THF) were purchased from Pharmaco (HPLC grade), dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) were purchased from Aldrich (HPLC grade). Deionized water (DI, 18.2 M Ω ·cm purity) was obtained from a RIOS 16/MILLQ synthesis Millipore water purification system, L-pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide (PFLNA) chromogenic substrate from Bachem. All chemicals were purchased in the highest available purity and were used as received except when otherwise specified.

Methods. *Determination of Hydrolytic Activity: Casein Assay/L-Pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide (PFLNA) Assay.* Procedure for the casein assay was performed as described in a previous reference by Geng Li et al.³¹ Hydrolytic assay using L-pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide was adapted from the method published by Filippova et al.³² In summary, to 2.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) containing 0.3 M KCl was added 0.3 mL of a 5 mM L-pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide solution (prepared in the desired cosolvent; MeOH, DMSO, DMF). After 5 min incubation at 37 °C, the enzyme solution (0.2 mL, 600 μ g protease/mL) in the same buffer was added, mixed well, and incubated at 37 °C. After 2 h, the reaction was terminated by addition of 0.2 mL 3N HCl solution and the OD₄₁₀ was measured in a Molecular devices spectramax plu384 (UV-vis) spectrophotometer. For blank experiments, the enzyme solution was added after addition of 3 N HCl. Further details on the assay method are given in Supporting Information.

General Procedure for Protease-Catalyzed Oligo(L-Phe) Synthesis. The method for oligo(L-Phe) synthesis was adapted from a literature procedure reported by Geng Li et al.^{17,31} In summary, L-Et-Phe·HCl (143.7 mg, 0.625 mmol), protease (16 units/mL), and 5 mL of 0.25 M sodium phosphate buffer solution set at a predetermined pH (commensurate with the optimum pH for oligo(γ -Et-L-Glu) synthesis for the protease used³¹) and a requisite amount of water-miscible cosolvent, for example (20% methanol v/v), was transferred to a 50 mL borosilicate glass tube, fitted with a Teflon cap, and placed in a parallel reactor Carousel 12 (Radleys discovery technologies). Reactions were performed with gentle magnetic stirring at 40 °C for 3 h. The reaction

mixture was cooled to room temperature, the resulting precipitated product was centrifuged, and the supernatant was discarded. The precipitate was then washed first with DI water (2×5 mL), then with an HCl solution (pH 2, 2×5 mL), and the remaining solid was lyophilized. Control experiments performed with substrates without addition of enzyme did not yield any precipitate.

Instrumental Methods. Nuclear Magnetic Resonance (NMR). ^1H NMR spectra was recorded on a Bruker DPX 300 spectrometer at 300 MHz. NMR experiments were performed in deuterated Dimethyl sulfoxide ($\text{DMSO}-d_6$), 1:1 deuterated chloroform CDCl_3 and trifluoroacetic acid (CF_3COOH) containing 10 mg/mL peptide, a total of 128 scans was recorded. Data was collected and analyzed by MestReC software. Proton chemical shifts were referenced to tetramethylsilane (TMS) at 0.00 ppm.

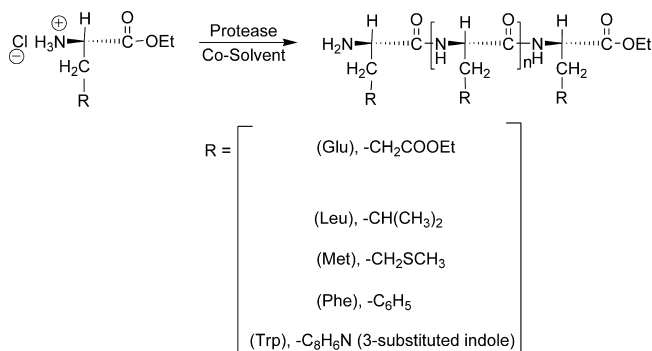
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF). MALDI-TOF spectra were obtained on an OmniFlex MALDI-TOF mass spectrometer (Bruker Daltonics Inc.) The instrument was operated in a positive ion reflector mode with an accelerating potential of +20 kV. The TOF mass analyzed has a pulsed ion extraction. The linear flight path is 120 cm. OMNIFLEX TOF control software is used for hardware control and calibration. The spectrum was obtained by averaging at least 300 laser shots. The pulsed ion extraction delay time was set at 200 ns. The spectrometer was calibrated using Angiotensin II as the external standard (1046.54 amu). To generate the matrix solution a saturated solution of α -cyano-4-hydroxycinnamic acid (CCA) was prepared in a 0.1% TFA [(0.5:2) water/acetonitrile] TA solution. Oligopeptide samples were dissolved in (0.1% TFA) dimethylsulfoxide solution (20 μL), and this solution was diluted with 230 μL of TA solution for a final concentration of 30 pmol/ μL (analysis mixture). A total of 15 μL of the (analysis mixture) sample solution was mixed with 15 μL of CCA (matrix) solution in a 100 μL eppendorf tube. A 0.5 μL aliquot of the sample and CCA mixture was applied to the steel target. The sample target was dried in ambient air. The abundance intensities of peaks m/z were collected via X-massOMNIFLEX6.0.0 and analyzed. The molecular weights obtained by experimental data were compared to a theoretical database created in MS Excel for the different chain lengths.

Results and Discussions

The formation of a heterogeneous reaction medium was observed prior to addition of protease when attempting to perform protease-catalyzed oligomerizations of L-Et-Phe·HCl (0.625 mmol) at 40 °C in 5 mL of 0.25 M sodium phosphate buffer solution (pH 8–9). Heterogeneous reactions complicate analysis of oligomerization reaction kinetics as well as core-activity when two or more monomers are substrates. Furthermore, these results suggest that studies with other hydrophobic amino acids will meet a similar fate. This prompted us to investigate the addition of water-miscible cosolvents that would enable the study of protease-catalyzed oligomerization of hydrophobic monomers under homogeneous conditions (Scheme 1). Concentration of water miscible solvents like DMSO, DMF, ACN, methanol, and ethanol above 50% v/v are known to deactivate the catalytic activity of α -chymotrypsin.¹⁵ Thus, to avoid protease deactivation, the initial part of this paper focuses on establishing minimum cosolvent concentrations required for monomer dissolution and to determine the extent that these cosolvents influence the activity of bromelain, papain, and α -chymotrypsin for conversion of L-Et-Phe·HCl and other hydrophobic amino acids to their corresponding homo-oligopeptides.

Effect of Buffer/Cosolvent Concentration. Prior to determining the catalytic efficiency of the proteases toward oligo(L-Phe) synthesis, an experiment was performed to determine the maximum concentration of water miscible cosolvent that would

Scheme 1. Oligomerization of L-Amino Acid Ethyl Ester Monomers by Protease Catalysis in Media Consisting of Aqueous Buffer–Water Miscible Cosolvent Systems



not precipitate sodium phosphate buffer salts from pH 8.0 solutions. Because the ability to use relatively high buffer concentration is desirable to maintain the pH at optimum values during protease-catalyzed oligomerization reactions,^{17,33} the influence of buffer concentration (0.25, 0.45, and 0.9 M) was also evaluated. Figure 1 illustrates that the differing solvating properties of DMSO, DMF, EtOH, MeOH, THF, and ACN results in dissimilar capacities of corresponding aqueous/cosolvent solutions to maintain sodium phosphate buffer salts in solution. Furthermore, for all cosolvents, increasing the buffer concentration decreases the maximum amount of cosolvent that can be added to solutions without salt precipitation. At the extremes, MeOH and THF can be added at the highest and lowest concentrations, respectively, regardless of the buffer concentration. For example, at 0.9 M buffer, amounts of MeOH and THF that can be added without salt buffer precipitation are 30 and 13%, respectively. At the lowest buffer concentration (0.25 M), up to 30% DMSO and DMF and up to 45% ethanol, methanol, and ACN could be added without salt precipitation. In contrast, the maximum content of THF that could be added at 0.25 M buffer is 28%.

To determine the minimum quantity of each cosolvent required to obtain a homogeneous solution, mixtures of L-Et-Phe·HCl (0.125 M) in 0.25 M sodium phosphate buffer (pH 8, 40 °C) were titrated with selected cosolvents. Resulting values of minimum cosolvent concentrations are shown on the x -axis of Figure 2. Experiments with THF showed that its addition up to 28%, where buffer salts precipitate, did not solubilize L-Et-Phe·HCl in the cosolvent medium. Hence, THF was not used in subsequent experiments. The relative influence of each cosolvent on % yield of precipitated oligo(L-Phe) is given in Figure 2. Reactions were conducted for 3 h at optimum pH

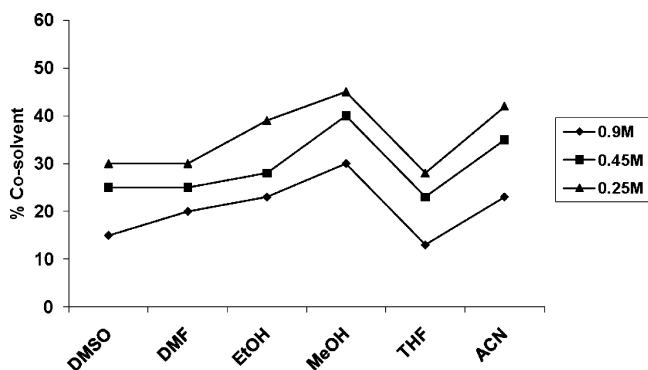


Figure 1. Plot depicting the maximum cosolvent concentrations that can be added to 0.9, 0.45, and 0.25 M sodium phosphate buffer solutions without precipitation of buffer salts.

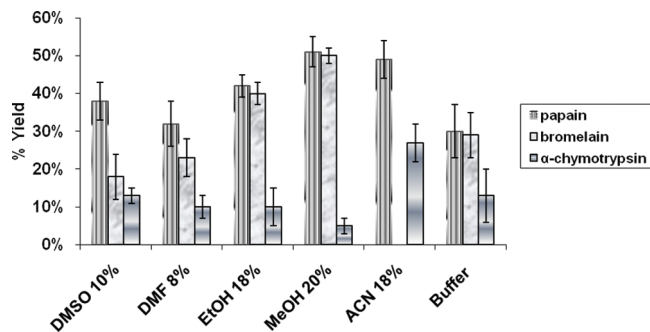


Figure 2. Protease-catalyzed L-Et-Phe·HCl oligomerization performed using the minimum cosolvent concentration (see values on the x-axis) that give homogeneous reaction media. Reaction conditions were as follows: (i) papain, bromelain, and α -chymotrypsin concentrations were 20.0, 18.6, and 16.6 mg/mL, respectively, (ii) 0.25 M sodium phosphate buffer, (iii) 0.125 M L-Et-Phe·HCl, (iv) 40 °C, (v) 3 h, and (vi) pH 8.0. Error bars represent the deviation from the mean of duplicate experiments.

values of 8, 8, and 9 for papain, bromelain, and α -chymotrypsin, respectively.³¹

Regardless of the solubilizing cosolvent selected, papain catalysis results in relatively high yields that ranged from 38% in 10% DMSO to 50% in both 20% MeOH and 18% ACN. In contrast, solvent selection has an extraordinary effect on bromelain-catalyzed oligo(L-Phe) yield. That is, no precipitated oligo(L-Phe) was obtained in 18% ACN, but in 10% DMSO, 8% DMF, 18% EtOH, and 20% MeOH the yields were 18, 20, 40, and 50%, respectively. Product yield for α -chymotrypsin was highest (27%) in 18% acetonitrile but, otherwise, was $\leq 10\%$. Even though papain-, bromelain-, and α -chymotrypsin-catalyzed oligomerizations in buffer without water-miscible cosolvent are heterogeneous, they still lead to the formation of oligo(L-Phe) (30, 29, and 13%, respectively). While performing α -chymotrypsin-catalyzed oligo(L-Phe) synthesis in selected cosolvents showed no substantial benefit with respect to product yield, large improvements in oligo(L-Phe) yield by addition of selected cosolvents were observed (see Figure 2). For example, with the addition of 20% MeOH relative to no cosolvent, the yield increased from ~ 29 to 51% and 50% for papain- and bromelain-catalyzed reactions.

Structural Analysis. The ¹H NMR spectrum of precipitated oligo(L-Phe), synthesized by bromelain catalysis (18.6 mg/mL) in 0.25 M phosphate buffer (40 °C, 3 h) at pH 8.0 with 20% MeOH v/v is displayed in Figure 3. Assignments other than the methine protons were based on those given in the literature for Boc-NH(L-Phe)₃-COOEt.³⁴ To assign the methine protons at terminal and internal positions along chains, a 2D NMR (¹H–¹H COSY90) was performed (see Figure S1 in Supporting Information). C-Terminal methine proton *d* (-CH-COO[H/Et]) at 4.54 correlated to proton *h* (-OC-NH-CH-COO[H/Et]) at 7.35 and methylene protons *b* -CH₂(C₆H₅) at 3.2. At the N-terminal methine proton *f* (H₃N-CH-CO-) at 4.95 correlated to -NH₃ (*h'*) proton at 7.63 and methylene protons *b* -CH₂(C₆H₅) at 3.13, the main chain methine protons *e* (-HN-CH-CO-) at 4.79 correlated to (-NH-CH-) at 7.93 and methylene protons *b* -CH₂(C₆H₅) at 2.92. The average degree of polymerization (DP_{avg}) was determined by the relative integration values of C- and N-terminal methine protons (*d* and *f*, respectively) and internal methine protons *e* occurring at 4.54, 4.95, and 4.79 ppm, respectively. For oligo(L-Phe) synthesized under these conditions, the DP_{avg} is 8.2 \pm 0.5. From analysis of DP_{avg} by ¹H NMR of oligo(L-Phe) synthesized over the full range of cosolvent and protease conditions described in Figure 2, the

variation in DP_{avg} between these samples was not significant and ranged between 7.5–8.3. This can be explained by that, the magnitude of differences in medium polarity as a function of the cosolvent used was not sufficient to alter the minimum hydrophobicity index required to cause precipitation of growing oligo(L-Phe) chains.

The MALDI-TOF spectrum displayed in Figure 4 is of the identical product characterized above by ¹H NMR. Three series of ion peaks, separated by 147 *m/z* units, equal to the mass of (L-Phe) repeat units, are observed and the peaks are isotopically resolved. Mass peaks corresponding to DP values 4–7 are shown in Figure 4. The most abundant signals are at DP 4 and 5. Because the DP_{avg} determined by ¹H NMR analysis is 8.2 \pm 0.5, it follows that MALDI-TOF analysis underestimates oligo(L-Phe) DP_{avg}. Indeed, it is well-known that MALDI-TOF signal intensities can vary to a large extent as a function of molecular structure due to differences in the extent of desorption from the matrix and molecular fragmentation of components in mixtures.^{35–37} The major series of signals corresponding to molecular ions *m/z* F₄(636, 657, 673), F₅(783, 804, 820), F₆(951, 967) and F₇(1085, 1098), separated by 147 *m/z* and associated with one H⁺, Na⁺, and K⁺ ion, respectively, are observed. Thus, results of MALDI-TOF suggest that the product is a mixture of different chain lengths of oligo(L-Phe). This heterogeneity can be rationalized by considering a minimum hydrophobicity index of growing oligo(L-Phe) chains which renders it insoluble in the reaction medium.

Effect of Substrate Concentration. Figure 5 displays the influence of substrate concentration on bromelain catalyzed oligo(L-Phe) synthesis conducted at 40 °C for 3 h with 0.25 M phosphate buffer (pH 8) and 20% MeOH (v/v). The product yield increased from 0 to 40% \pm 4% and 50% \pm 5% by increasing the monomer concentration from 0.02 to 0.05 and 0.09M, respectively. A further increase in substrate concentration from 0.09 to 0.16 M resulted in no substantial change in product yield. Even at relatively low monomer concentrations (e.g., 0.05 M, 1.2% by wt), appreciable yields (40%) of oligo(L-Phe-Et) is formed. This agrees with previous work by our group for papain-catalyzed L-Glu-Et oligomerization³¹ and reveals that oligomerization kinetics are sufficiently rapid allowing product formation without requiring high substrate concentrations to shift the equilibrium toward product.¹⁷ With increased substrate concentration and constant product yield there is increased volumetric product formation and, therefore, more HCl liberated. For example, the volumetric yield of oligo(L-Phe) at 0.05 and 0.16 M L-Phe-Et is 3.6 and 11.4 g/L, respectively. By moving toward higher L-Phe-Et concentrations that give higher volumetric yields there is the potential due to HCl formation of causing decreased reaction pH that results in reduced volumetric yields. However, pH decreased from 8.0 to 7.4 during the course of L-Phe-Et oligomerizations with initial monomer concentration 0.14 M, and the pH must decrease below 7 to decrease bromelain activity during L-Phe-Et oligomerizations (see Figure 9). Therefore, at least under this range of buffer and monomer concentrations, increased monomer concentrations did not decrease product yield. The DP_{avg} (¹H NMR) showed no appreciable change as a function of substrate concentration. This is reasonable since substrate concentrations are not sufficiently high to appreciably affect oligo(L-Phe) solubility.

A time course study with 20% MeOH was performed to determine the progress of product formation over 3 h reactions. The time-course plot, displayed in Figure 6, shows that in only 15 min oligo(L-Phe) yield reached 23% \pm 4%. Thereafter, the oligo(L-Phe) yield increased more slowly reaching yields of 31%

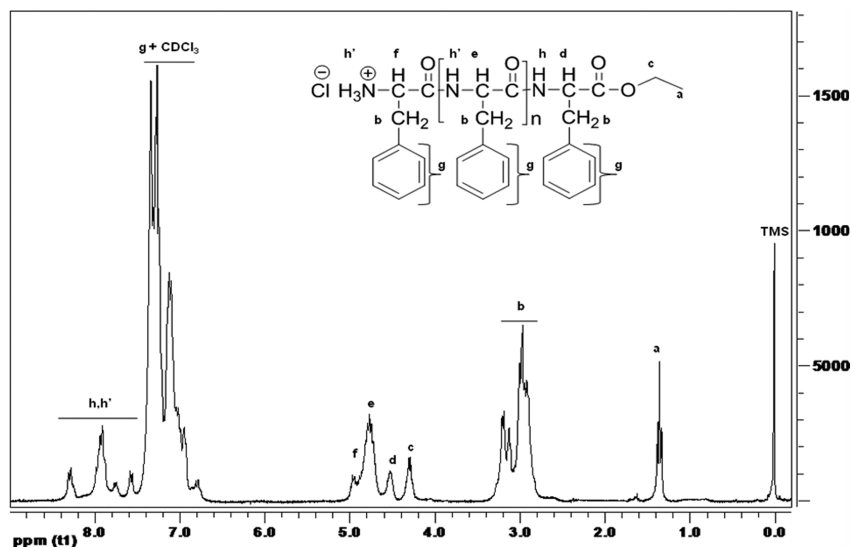


Figure 3. ¹H NMR (300 MHz, 1:1 TFA/CDCl₃) spectrum of oligo(L-Phe) synthesized using 0.125 M L-Et-Phe·HCl, 18.6 mg/mL bromelain, 0.25 M phosphate buffer, at 40 °C, for 3 h, using 20% MeOH (v/v) at pH 8.

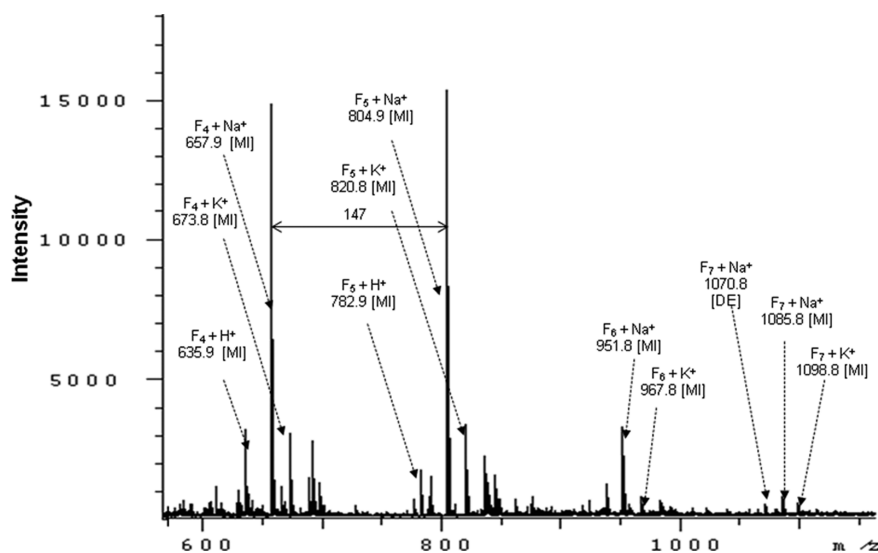


Figure 4. MALDI-TOF spectrum of oligo(L-Phe) synthesized using 0.125 M L-Et-Phe·HCl, 18.6 mg/mL bromelain, 0.25 M phosphate buffer containing 20% MeOH (v/v), at 40 °C, for 3 h, at pH 8. F is the common 1-letter abbreviation used for phenylalanine. [DE] represents desterified, [M] represents molecular ion peak, the *m/z* values are ± 1 da of the expected *m/z* values.

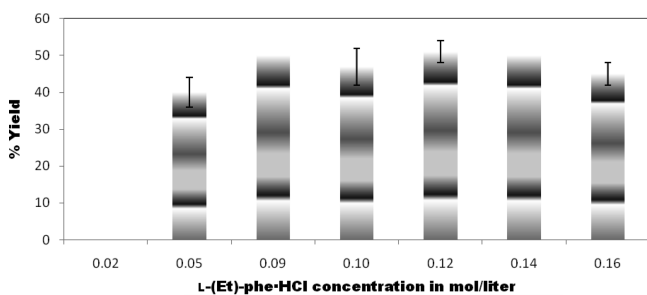


Figure 5. Effect of substrate concentration on oligo(L-Phe) % yield. Reactions were conducted at 40 °C for 3 h using 18.6 mg/mL bromelain, 0.25 M phosphate buffer, with 20% MeOH (v/v) at pH 8. Error bars represent the deviation from the mean of duplicate experiments.

$\pm 5\%$ and $43\% \pm 4\%$ in 60 and 120 min, respectively. Further increase in the reaction beyond 120 min shows no substantial increase in product yield. Hence, about 40% of total product formed within the first 15 min and, thereafter, the reaction slows. One explanation for these results is bromelain deactivation. Indeed, it is well-known that proteases undergo autolysis during

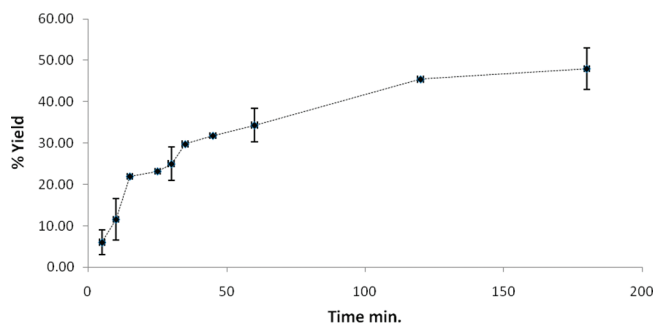


Figure 6. Plot of reaction time vs oligo(L-Phe) % yield. Reactions were conducted at 40 °C using 18.6 mg/mL bromelain, 0.25 M phosphate buffer, with 20% MeOH (v/v) at pH 8. Error bars represent the deviation from the mean of duplicate experiments.

reactions in aqueous media.²⁸ Alternatively, the hydrolysis of L-Phe-Et ester groups or a fraction of C-terminal unit ethyl ester moieties on oligo(L-Phe) can result in formation of shorter chain oligomers that do not precipitate both slowing product formation and ultimately limiting yields of precipitated oligopeptide. In

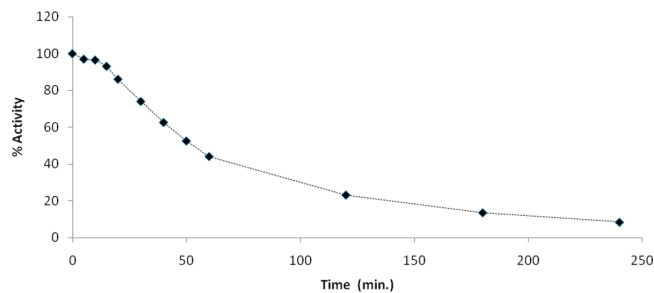


Figure 7. Plot of bromelain activity, determined using the chromogenic substrate L-pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroaniliden (PFLNA) vs incubation time.

comparison to papain-catalyzed oligo(γ -Et-Glu) synthesis where yields of precipitated product reached >80% in 20 min,¹⁷ bromelain-catalyzed oligo(L-Phe) synthesis in the presence of 20% methanol (v/v) is slow reaches a plateau at about 2 h where yields do not exceed 42% \pm 5%. Furthermore, without methanol addition to reaction media the yield obtained after 3 h was 29% \pm 4% (see Figure 2).

A study was performed to test the hypothesis given above that bromelain deactivation was responsible for the slowing of oligo(L-Phe) synthesis after 15 min where the precipitated product yield was only 23% \pm 4%. Bromelain was incubated at 40 °C in 0.25 M sodium phosphate buffer (pH 8) containing 20% v/v MeOH. Periodically, aliquots of this solution were taken and bromelain hydrolytic activity was analyzed using the chromogenic substrate L-pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide (PFLNA). The results in Figure 7 show that bromelain loses 85% of its activity during a 3 h incubation time. Initially, activity loss versus time is slow (slope = -0.0045) so that, at 15 min, bromelain activity decreased by 17%. Thereafter, the negative slope of % activity versus time increased to (-0.016) so that, at 50 min, bromelain activity decreased by 50%. Therefore, these results support that bromelain deactivation during the course of reactions contributes substantially to the observed decrease in precipitated product yield after only 15 min.

Effect of Enzyme Concentration. Enzyme concentrations used for reactions in preceding sections was based on literature values from a previous study on papain-catalyzed oligo(γ -Et-Glu) synthesis.³¹ The strategy employed by our laboratory to correlate protein concentrations for different protease catalysts used in oligopeptide synthesis has been to measure protein content and activity of as-received enzyme preparations by the BCA method and casein hydrolysis assay, respectively. Then, protein concentrations are normalized based on the number of activity units where a unit is the quantity of protease (determined by BCA) required to release 1 μ mol of tyrosine equivalents per min per mL in buffer (pH 7.5) at 37 °C. For example, for the above studies that compared papain, bromelain, and α -chymotrypsin, 16 units of each enzyme was used.³¹ Clearly this method of normalization of activity between different proteins has flaws. For instance, casein hydrolytic activity may not correlate with a protein's activity for peptide synthesis and protein preparations differ in their level of purity. Given the above, bromelain-catalyzed oligomerization of L-Phe-Et, carried out in a mixed solvent system consisting of 20% v/v MeOH and 0.25 M phosphate buffer (pH 8), was investigated for 3 h reactions. Figure 8 shows that, by increasing the bromelain concentration from 4 to 18.6 mg/mL, a regular increase in the product yield from 6 to 43% \pm 4% was observed. A further increase in enzyme concentration from 18.6 to 20 mg/mL resulted in no significant change in product yield. Hence, 18.6 mg/mL bro-

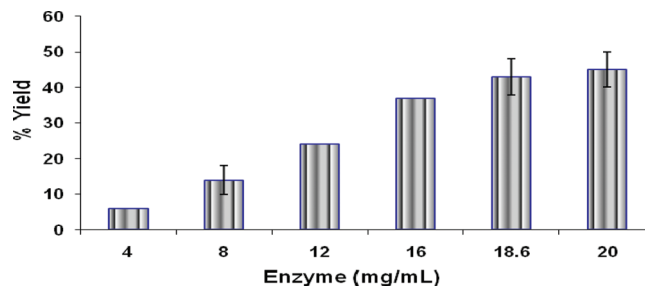


Figure 8. Plot of bromelain concentration vs oligo(L-Phe) % yield. Reactions were conducted for 3 h, at 40 °C, in 0.25 M phosphate buffer (pH 8) with 20% MeOH (v/v). Error bars represent the deviation from the mean of duplicate experiments.

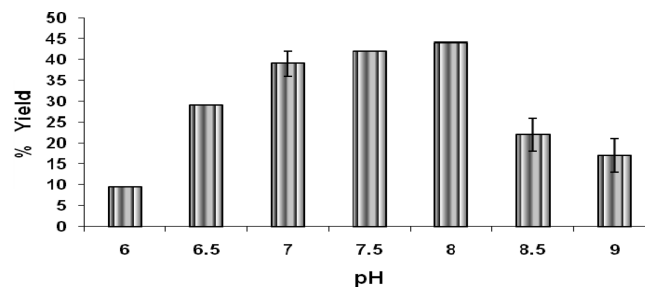


Figure 9. Effect of initial reaction pH on oligo(L-Phe) % yield. Reactions were conducted for 3 h, at 40 °C, using 18.6 mg/mL bromelain in 0.25 M phosphate buffer and 20% MeOH (v/v). Error bars represent the deviation from the mean of duplicate experiments.

melain that correlates to 16 activity units, was selected for experiments below with bromelain in 20% (v/v) MeOH. It follows that the optimum bromelain concentration derived from oligo(γ -Et-Glu) synthesis at pH 8.0¹⁷ correlates well with oligo(L-Phe) synthesis. Thus, even though the normalization method is flawed, it provides an initial basis for selecting protease quantities for oligopeptide synthetic transformation.

Effect of pH on Synthesis. The bromelain pH value of 8.0, used for studies in preceding sections of this paper, was based on a literature value from bromelain-catalyzed oligomerization of oligo(γ -Et-Glu) synthesis in 0.9 M phosphate buffer at 40 °C.³¹ Therefore, the optimal pH was reinvestigated for oligo(L-Phe) synthesis in 0.25 M phosphate buffer (pH 8) with 20% v/v MeOH. Study of Figure 9 shows that, maximum product yields, between 40 and 45%, were obtained for initial pH values from 7 to 8. Since the pH of reaction media can decrease as reactions proceed, pH values at the end of reactions were recorded. Changes in pH were as follows: 6.0 to 5.0, 6.5 to 5.5, 7.0 to 6.4, 7.5 to 6.9, 8.0 to 7.2, 8.5 to 8.0, and 9.0 to 8.5. Thus, pH changes during reactions were substantial and ranged from 0.5 to 1 pH unit. The fact that the pH of the reaction at 7.0 decreased to 6.4 suggests that, if the pH were kept constant throughout reactions, the pH range for maximum product yields would extend from \sim 6.5 to 8.0. Analysis by ¹H NMR showed that the DP_{avg} of products formed at initial pH values between 6.0 and 9.0 ranged from 7.6 to 8.4. Furthermore, analysis of these products by MALDI-TOF revealed that the distribution of chain lengths from 4–7 mer remained unchanged. Thus, there was no apparent change in oligo(L-Phe) DP_{avg} nor its chain-length distribution. In comparison to the above, papain, when catalyzing L-Et₂-Glu oligomerizations, has a broader range of pH values that give high product yields (5.5 to 8.5), and maximum product yields were higher (70–75%).³¹

Effect of Temperature. Studies above on bromelain-catalyzed L-Phe-Et oligomerizations were conducted at 40 °C

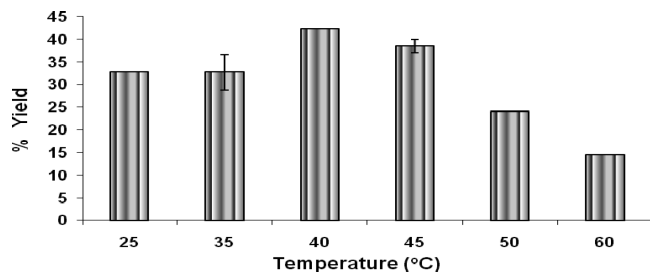


Figure 10. Influence of reaction temperature on oligo(L-Phe) % yield. Reactions were conducted using 18.6 mg/mL bromelain in 0.25 M phosphate buffer (pH 8) and 20% MeOH (v/v). Error bars represent the deviation from the mean of duplicate experiments.

based on temperatures commonly used in the literature for bromelain-catalyzed hydrolysis reactions.³⁸ To the best of our knowledge, the temperature dependent activity of bromelain for oligopeptide synthesis from L-amino acid ester monomers has not previously been reported. Inspection of Figure 10 shows that, as the temperature at which reactions were conducted was increased from 45 to 50 and 60 °C, the % yield of oligo(L-Phe) synthesis decreased from 38 to 24 and 14%, respectively. Given that the % yield appears slightly higher at 40 than 45 °C (44 vs 38), 40 °C was taken as the preferred temperature. This was a fortunate happenstance given our selection of this temperature for experiments above. The interpretation of why an increase in reaction temperatures above 45 °C dramatically decreased yield is not straightforward. It may be that increasing reaction temperature increases the rate of ester hydrolysis relative to amide synthesis, which would result in decreased oligopeptide yield. Furthermore, a study of bromelain denaturation is complicated by the fact that the enzyme preparation used is impure (see Materials section). Study of the oligo(L-Phe) products obtained at temperatures between 25 and 60 °C by ¹H NMR showed no substantial change in their DP_{avg} values. Correspondingly, MALDI-TOF analysis of these same products showed similar relative intensities of peaks corresponding to 5-, 6-, and 7mers. Hence, any potential change in oligopeptide solubility was not manifested in its DP_{avg} or chain length distribution.

Influence of MeOH Cosolvent on Other Amino Acid Oligomerizations. To further build on our understanding of bromelain-catalyzed amino acid oligomerizations in the presence of water-miscible cosolvents, a series of experiments were conducted in 0, 20, and 30% MeOH. Of interest was to determine how (i) the outcome of oligomerizations is influenced by moving from heterogeneous to homogeneous reaction conditions, (ii) increases in MeOH concentration beyond that required for monomer solubilization influence oligomerization, and (iii) MeOH content in reaction media affects oligomerization of other amino acid ethyl ester monomers.

By conducting bromelain-catalyzed oligo(L-Phe) oligomerizations in the absence of MeOH, the product yield is 29%. Addition of 20% MeOH to solubilize 0.125 M L-Phe-Et in 0.25 M phosphate buffer (pH 8.0) resulted in an increase in oligo(L-Phe) yield to 51% ± 4%. However, further increasing the MeOH concentration beyond that required to achieve a homogeneous reaction medium resulted in a small but substantial decrease in % yield to 44% ± 4%. Thus, while providing sufficient MeOH to achieve homogeneous reaction conditions proved beneficial to product yields, a further increase in MeOH concentration can have negative effects on product yields presumably due to decreased enzyme activity. Negative effects on enzyme activity by addition of various water-miscible cosolvents has been observed with a wide range of enzyme families.³⁹

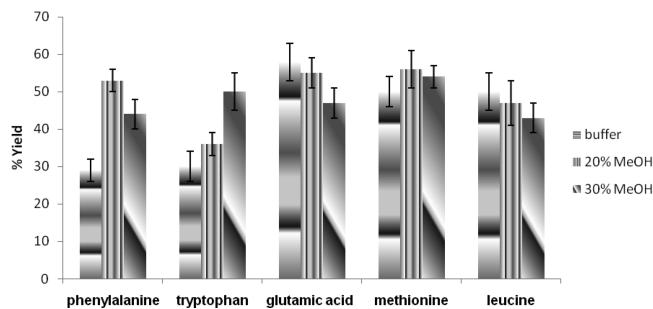


Figure 11. Influence of MeOH concentration and amino acid ethyl ester monomer used on % yield of precipitated oligomers. Reactions were conducted at 40 °C using 18.6 mg/mL bromelain in 0.25 M phosphate buffer (pH 8). Error bars represent the deviation from the mean of duplicate experiments.

Similar to L-Phe-Et, attempts to solubilize 0.125 M L-Trp-Et at 40 °C in 0.25 M phosphate buffer (pH 8.0) resulted in a heterogeneous reaction medium. Bromelain-catalyzed oligomerization of L-Trp-Et without MeOH addition gave oligo(L-Trp) in 30% ± 3% yield. Subsequently, oligomerizations were performed in reaction media containing 20 and 30% v/v MeOH. By addition of 20% MeOH to the reaction medium, turbidity measurements proved that L-Trp-Et solubility increased. However, the reaction medium remained turbid. Oligomerization of L-Trp-Et with 20% MeOH resulted in an increase in yield of up to 6%. Addition of 30% MeOH was required to solubilize 0.125 M L-Trp-Et at 40 °C in 0.25 M phosphate buffer (pH 8.0). Under these conditions, the yield of oligo(L-Trp) was 50% ± 4%. ¹H NMR analysis of oligo(L-Trp) (see Supporting Information, Figure S2) showed that the DP_{avg} of products formed in 0, 20, and 30% MeOH is 5, 6, and 6, respectively. Furthermore, with increased cosolvent concentration MALDI-TOF spectra (see Supporting Information, Figure S3) of oligo(L-Trp) products were alike indicating that changes in the cosolvent concentration had no apparent effect on oligo(L-Trp) molecular weight distribution. That is, relative intensities of the most abundant MALDI-TOF peaks observed at 627, 813, and 1000 *m/z*, corresponding to molecular ions associated with one sodium ion for 3, 4, and 5 unit oligo(L-Trp) chains, did not substantially change.

For oligomerizations of 0.125 M concentrations of L-Et₂-Glu, L-Met-Et, and L-Leu-Et, there was no need to add MeOH to solubilize these monomers in 0.25 M phosphate buffer (pH 8.0). However, co-oligomerizations of these monomers with hydrophobic monomers L-Trp-Et and L-Phe-Et may require the presence of a water-miscible cosolvent to achieve homogeneous reaction media. Furthermore, because L-Et₂-Glu, L-Met-Et, and L-Leu-Et are all soluble without cosolvent, these monomers enable an unambiguous determination of how MeOH addition to reaction media affects oligomer yields and chain lengths. Study of Figure 11 shows there is no apparent effect on oligo(γ-L-Et-Glu), oligo(L-Met), and oligo(L-Leu) yields by addition of 20% MeOH to buffer solutions. While increasing the MeOH concentration to 30% had no effect on oligo(L-Met) yield, a significant but small decrease in oligo(γ-L-Et-Glu) yield was observed when methanol concentration was increased from 20 to 30% (55 to 47%). Furthermore, a significant but small decrease in oligo(L-Leu) yield was observed when comparing 0 and 30% MeOH (50 to 43%). Overall, concentrations of up to 30% MeOH are well tolerated by bromelain, at least with respect to its activity for oligomerization of amino acid ethyl esters at 40 °C in 0.25 M phosphate buffer pH 8. Observation of MALDI-TOF spectra recorded for oligopeptides synthesized

in the presence of different MeOH concentrations (see Figure 11) showed no appreciable changes in the distribution of chain lengths. This result is reasonable since oligo(L-Phe), oligo(L-Trp), oligo(γ -L-Et-Glu), oligo(L-Met), and oligo(L-Leu) are all insoluble in MeOH. In other words, MeOH would not substantially enhance the solubility of these peptides, which largely determines the molecular weights reached. MALDI-TOF spectra recorded of oligo(L-Trp), oligo(γ -L-Et-Glu), oligo(L-Met) and oligo(L-Leu) synthesized with 30% MeOH are displayed in the Supporting Information (see Figures S3, S5, S7, and S9, respectively). MALDI-TOF spectra of these oligopeptides reveal that they all consist of a wide distribution of chain lengths. For example, mass peaks corresponding to 7- to 12mer units were observed for oligo(γ -L-Et-Glu). Furthermore, over the entire range of cosolvent concentrations, the highest intensity peaks were observed for oligo(γ -L-Et-Glu) with 7- and 8mer units. Also, mass peaks corresponding to 6- to 10mer units were observed for oligo(L-Met), and over the entire range of cosolvent concentrations, the highest intensity peaks were observed for oligo(L-Met) with 6- and 7mer units. For oligo(L-Leu), mass peaks were observed corresponding to 5- to 8mer units and, over the entire range of cosolvent concentrations, the highest intensity peaks were found for oligo(L-Leu) with 6- and 7mer units. DP_{avg} determined by 1H NMR also showed no significant variations for oligopeptides synthesized with different MeOH concentrations shown in Figure 11. 1H NMR spectra recorded of oligo(L-Phe), oligo(L-Trp), oligo(γ -L-Et-Glu), oligo(L-Met), and oligo(L-Leu) synthesized with 30% MeOH are displayed in the Supporting Information (see Figures S1, S2, S4, S6, and S8, respectively). Values of DP_{avg} for oligo(γ -L-Et-Glu),¹⁷ oligo(L-Met),³⁰ and oligo(L-Leu)³¹ are 8.2 ± 0.6 , 8 ± 0.8 , and 7.9 ± 0.4 , respectively.

Influence of DMSO and DMF on DP_{avg} of Oligo(L-Phe). DMF and DMSO can dissolve oligo(L-Phe) with DP_{avg} and chain length distributions as described above. Thus, if the protease catalyst can tolerate sufficiently high concentrations of DMSO or DMF, it should then follow that precipitated oligo(L-Phe) with higher chain lengths will be obtained. To investigate this possibility, concentrations of DMSO and DMF of 10, 20, and 30% v/v were investigated as cosolvents for bromelain-catalyzed L-Phe-Et oligomerizations performed at 40 °C in 0.25 M phosphate buffer (pH 8). A total of 10% cosolvent is the minimum concentration of DMF and DMSO required to solubilize 0.125 M L-Et-Phe·HCl at pH 8.0. A total of 30% cosolvent is the maximum concentrations of DMF and DMSO that can be added to reactions without precipitation of buffer salts. Unfortunately, 30% concentrations of DMF and DMSO do not appear to sufficiently alter the solvent characteristics so that only small increases in oligo(L-Phe) DP_{avg} (from 8.2 to 9) was observed. MALDI-TOF spectra of products over the entire range of DMF and DMSO concentrations showed no apparent change in mass peak distribution.

The exploration of DMF and DMSO concentrations above 30% with lower buffer salt concentrations would require that these solvents do not greatly decrease enzyme activity. However, the yield of the precipitated oligo(L-Phe) decreased from 20 to 4% for DMSO concentrations of 10 and 30%, respectively. Similarly, the yield of precipitated oligo(L-Phe) decreased from 25 to 5% for DMF concentrations of 10 and 30%, respectively. Thus, based on decreased product yields, it appears that 20 and 30% concentrations of DMF and DMSO results in bromelain denaturation decreasing its activity. Results above on oligopeptide synthesis in MeOH containing reaction media showed that 30% MeOH was well tolerated by bromelain. To directly

quantify changes in bromelain activity as a function of the cosolvent structure and concentration, an assay was performed using the chromogenic substrate L-pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide (PFLNA).³² Given that the molar extinction coefficient (ϵ) of *p*-nitroaniline will be solvent dependent, *p*-nitroaniline ϵ -values were determined in buffer cosolvent mixtures (10, 20, and 30%, respectively) and the values obtained are given in the Supporting Information (Table S1). The activity at 0% cosolvent could not be measured accurately since the synthetic substrate is not soluble in the absence of cosolvents. This observation necessitated, equating the activity of bromelain at 10% v/v cosolvent ratio as equal to 100% activity. Designating the activity of bromelain at 10% cosolvent as 100%, bromelain activity at 20 and 30% DMSO concentrations decreased to 64 and 8%, respectively. Similarly, bromelain activity at 20 and 30% DMF decreased to 38 and 3%, respectively. In contrast, bromelain activity at 30%, relative to 20% MeOH concentration decreased to 57%, data given in Supporting Information (Table S2). Future work will address the need to identify proteases that retain their activity for oligopeptide synthesis in suitable reaction media that also solubilize desired oligopeptide structures thereby enabling the synthesis of higher chain length products.

Conclusions

This paper encountered and solved the following problem: oligomerizations of L-Et-Phe and other hydrophobic amino acid ethyl esters in aqueous buffer solution (pH 8) form heterogeneous mixtures prior to protease addition. Heterogeneous reactions are undesirable for maximizing reaction rates and for fundamental investigations such as kinetic studies. A solution is to utilize water-miscible cosolvents that minimize protease activity loss for amino acid alkyl ester oligomerizations. The minimum concentration of DMSO, DMF, EtOH, MeOH, and ACN that solubilize L-Phe-Et in sodium phosphate buffer salt media (pH 8.0, 40 °C) is 10, 8, 18, 20, and 18%, respectively. Between papain, bromelain, and α -chymotrypsin, the L-Phe-Et oligomerization activity of papain was least influenced by the choice of water-miscible cosolvent. Comparison of no cosolvent addition vs addition of 20% MeOH for bromelain and papain catalyzed oligo(L-Phe) synthesis resulted in increased precipitated product yields from 29% to 50% and 30% to 51%, respectively, signifying the benefit of transforming reactions from heterogeneous to homogeneous conditions.

A series of studies were performed in 20% MeOH phosphate buffer media to interrogate how the reaction pH, temperature, time, substrate concentration, and bromelain concentration influences bromelain oligo(L-Phe) synthesis activity. Furthermore, effects of reaction parameters on oligo(L-Phe) average chain length and distribution was determined by 1H NMR and MALDI-TOF, respectively. The cumulative results indicated that maximum product yields, between 40 and 45%, were obtained for initial pH values 7–8 and 40 °C was the preferred reaction temperature. It was further determined that concentrations up to 30% MeOH were well tolerated by bromelain. Attempts to study concentrations above 30% were not possible due to precipitation of media buffer salts.

Studies using DMF and DMSO as cosolvents for bromelain-catalyzed oligo(L-Phe) synthesis were performed to (i) determine bromelains activity/stability under such conditions and (ii) explore cosolvents that can enhance peptide solubility and, thereby, lead to higher chain length products. However, the yield of precipitated oligo(L-Phe) decreased to 4 and 5% in reactions

containing 30% DMSO and 30% DMF, respectively. Correspondingly, in reactions containing 30% DMSO and 30% DMF, bromelain activity decreased to 8 and 3% of its activity in 10% concentrations of these solvents.

The results above provide direction to researchers as to protease activity for oligopeptide synthesis as a function of water-miscible cosolvent structure and concentration. Given the potential of protease-catalyzed routes to provide oligopeptides with a wide range of functional and biological properties in combination with the poor solubility of many peptide structures in aqueous media, future work is needed to address the identification or engineering of proteases with improved stability, activity, and specificity for oligopeptide synthetic reactions.

Acknowledgment. The authors thank the National Science Foundation Industry/University Cooperative Research Center (NSF-IUCRC) for Biocatalysis and Bioprocessing of Macromolecules at Polytechnic Institute of NYU for their financial support.

Supporting Information Available. 2D NMR (^1H - ^1H COSY90) of oligo(L-Phe); ^1H NMR and MALDI-TOF spectra of products formed using the following amino acid ethyl ester hydrochlorides L-Trp-Et \cdot HCl, L-Et $_2$ -Glu \cdot HCl, L-Met-Et \cdot HCl, and L-Leu-Et \cdot HCl. Molar absorptivity coefficients (ϵ) for *p*-nitroaniline in MeOH, DMSO, and DMF at different cosolvent ratios. Hydrolytic activity measurements for bromelain in the presence of different ratios of water miscible cosolvents. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

- (1) Langer, R.; Tirrell, D. A. *Nature* **2004**, *428*, 487–492.
- (2) Lutolf, M. P.; Hubbell, J. A. *Nat. Biotechnol.* **2005**, *23*, 47–55.
- (3) Hartgerink, J. D.; Beniash, E.; Stupp, S. I. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5133–5138.
- (4) Reches, M.; Gazit, E. *Science* **2003**, *300*, 625–627.
- (5) Vauthey, S.; Santoso, S.; Gong, H.; Watson, N.; Zhang, S. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5355–5360.
- (6) Zompra, A. A.; Galanis, A. S.; Werbitzky, O.; Albericio, F. *Future Med. Chem.* **2009**, *1*, 361–377.
- (7) Schnolzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Res. Ther.* **2007**, *13*, 33–41.
- (8) Kent, S. B. H. *Annu. Rev. Biochem.* **1988**, *57*, 957–989.
- (9) Castelletto, V.; Hamley, I. W. *Biophys. Chem.* **2009**, *141*, 169–174.
- (10) Mayo, K. H. *Trends Biotechnol.* **2000**, *18*, 212–217.
- (11) Sadler, K.; Zeng, W.; Jackson, D. C. *J. Pept. Res.* **2002**, *60*, 150–158.
- (12) O'Brien-Simpson; Ede, N. M.; Brown, N. J.; Swan, L. E.; Jackson, J.; David, C. *J. Am. Chem. Soc.* **1997**, *119*, 1183–1188.
- (13) Hubbell, J. A. *Curr. Opin. Biotechnol.* **2003**, *14*, 551–558.
- (14) Maskarinec, S. A.; Tirrell, D. A. *Curr. Opin. Biotechnol.* **2005**, *16*, 422–426.
- (15) Jakubke, H. D.; Kuhl, P.; Könnecke, A. *Angew. Chem., Int. Ed.* **1985**, *24*, 85–93.
- (16) Schellenberger, V.; Jakubke, H. D. *Angew. Chem., Int. Ed.* **1991**, *30*, 1437–1449.
- (17) Li, G.; Vaidya, A.; Viswanathan, K.; Cui, J.; Xie, W.; Gao, W.; Gross, R. A. *Macromolecules* **2006**, *39*, 7915–7921.
- (18) Kobayashi, S.; Makino, A. *Chem. Rev.* **2009**, *109*, 5288–5353.
- (19) Koskien, A.; Klibanov, A. K. In *Enzymatic Reactions in Organic Media*, 1st ed.; Blackie Academic and Professional: London, 1996; p 332.
- (20) Schneider, M. P.; Lobell, M. *J. Chem. Soc., Perkin. Trans. 1* **1998**, 319–326.
- (21) Ye, Y. H.; Tian, G. L.; Xing, G. W.; Dai, D. C.; Chen, G.; Li, C. X. *Tetrahedron* **1998**, *54*, 12585–12596.
- (22) Bordusa, F. *Chem. Rev.* **2002**, *102*, 4817–4868.
- (23) Mozhaev, V. V.; Khmel'nitsky, Y. L.; Sergeeva, M. V.; Belova, A. B.; Klyachko, N. L.; Levashov, A. V.; Martinek, K. *Eur. J. Biochem.* **1989**, *184*, 597–602.
- (24) Kidd, R. D.; Sears, P.; Huang, D. H.; Witte, K.; Wong, C. H.; Farber, G. K. *Protein Sci.* **1999**, *8*, 410–417.
- (25) Szabó, A.; Kotormán, M.; Laczkó, I.; Simon, L. M. *J. Mol. Catal. B: Enzym.* **2006**, *41*, 43–48.
- (26) Homandberg, G. A.; Mattis, J. A.; Laskowski, M. *Biochemistry* **1978**, *17*, 5220–5227.
- (27) Barbas, C. F.; Matos, J. R.; West, J. B.; Wong, C. H. *J. Am. Chem. Soc.* **1988**, *110*, 5162–5166.
- (28) Whitesides, G. M.; Wong, C. H. *Enzymes in Synthetic Organic Chemistry*; Elsevier: New York, 1994; Vol. 12.
- (29) West, J. B.; Wong, C. H. *J. Org. Chem.* **1986**, *51*, 2728–2735.
- (30) Barbas, C. F.; Matos, J. R.; West, J. B.; Wong, C. H. *J. Am. Chem. Soc.* **2002**, *110*, 5162–5166.
- (31) Li, G.; Raman, V. K.; Xie, W.; Gross, R. A. *Macromolecules* **2008**, *41*, 7003–7012.
- (32) Filippova, I. Y.; Lysogorskaya, E. N.; Oksenoit, E. S.; Rudenskaya, G. N.; Stepanov, V. M. *Anal. Biochem.* **1984**, *143*, 293–297.
- (33) Uyama, H.; Fukuoka, T.; Komatsu, I.; Watanabe, T.; Kobayashi, S. *Biomacromolecules* **2002**, *3*, 318–323.
- (34) Adams, D. J.; Atkins, D.; Cooper, A. I.; Fuzzeland, S.; Trewin, A.; Young, I. *Biomacromolecules* **2008**, *9*, 2997–3003.
- (35) Albrethsen, J. *Clin. Chem.* **2007**, *53*, 852–858.
- (36) Dreisewerd, K. *Chem. Rev.* **2003**, *103*, 395–426.
- (37) Amado, F. M. L.; Domingues, P.; Santana-Marques, M. G.; Ferrer-Correia, A. J.; Tomer, K. B. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1347–1352.
- (38) Greenberg, D. M., Plant proteolytic enzymes. In *Methods Enzymology*; Academic Press: New York, 1955; Vol. 2, pp 54–64.
- (39) Castro, G. R.; Knubovets, T. *Crit. Rev. Biotechnol.* **2003**, *23*, 195–232.

BM100516X