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Protease Catalyzed In Situ C-Terminal Modification of Oligoglutamate

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

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

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ABSTRACT: One-pot biotransformations gave oligo(γ-L-Et-Glu) decorated with selected amine-functionalized end-groups at C-termini. Motivations for this work were to (i) control the end group structure of peptides synthesized by protease-catalyzed peptide synthesis and (ii) incorporate end-groups that can be used directly or after further modification as polymerizable entities. Papain, bromelain, α-chymotrypsin, Multifect P-3000, and Purafect prime 4000 L were used as catalysts for oligomerization of γ-L-$(Et)_2$-Glu in the presence of monofunctional amines. The series of amine nucleophiles (NH_2-R, acyl acceptors) studied mimic phenylalanine in that they possess aromatic rings linked to amine groups by one or more methylenes. Generally, addition of increased quantities of NH_2-R from 0 to 30, 50, and 70 mol % with respect to γ-L-$(Et)_2$-Glu results in decreased % yield, but increased mol % of NH_2-R end-capped oligo(γ-L-Et-Glu)-NH-R (determined by NMR). Irrespective of the protease used, 2-thiophene methyl amine (TPMA) gave the highest fraction of oligo(γ-L-Et-Glu)-NH-R chains. For example, using Multifect P-3000 and a feed ratio of TPMA- to γ-L-$(Et)_2$-Glu of 7:3, >90 mol % of oligopeptides formed had TPMA C-terminal groups. With all five proteases studied herein, L-phenylalanine and L-histidine did not produce end-capped oligo(γ-L-Et-Glu). In contrast, L-phenylalanine analogs benzylamine (BzA) and L-phenylalaninol (F-OH), both of which lack the α-carboxyl group, gave substantial quantities of oligo(γ-L-Et-Glu)-F-OH or -BzA chains. Hence, the results of this study prove that the promiscuity of proteases used herein can be exploited to create a diverse family of desired end-functionalized oligopeptides. MALDI-TOF spectra recorded of oligo(γ-L-Et-Glu) with amine nucleophiles showed molecular ions that affirmed the formation of corresponding NH_2-R functionalized oligo(γ-L-Et-Glu).

Introduction

Peptides are functionally rich, and their use in materials with properties such as pH-sensitivity, self-assembly, bioresorbability, and bioactivity are areas of intense interest. Often, peptide-based materials consist of amino acid sequences found in important natural proteins such as collagen, keratin, elastins, and silk.

Two-step routes to prepare peptides with terminal groups that can be polymerized or attached to surfaces represent important examples of research aimed at expanding the structure and function of natural peptides and proteins. For example, work was performed to mimic silk β-sheet elements in block copolymers.[1−3] Model oligo(lysine) and oligo(glutamic acid) functionalized polyphenylene dendrimers were prepared to study DNA complexation and as building blocks processed by electrostatic layer-by-layer self assembly to create supramolecular architectures.[4] Other research groups have designed peptides with cell adhesion properties [e.g., $(Arg\text{-}Ala\text{-}Asp\text{-}Ser)_n$, where $n = 2, 3$] at the N-terminus followed by a oligoalanine linker and a cysteine residue at the C-terminus for attachment onto gold surfaces. Peptides have also been modified at N-terminal units via acetylation or methylation to improve their stability in biological matrices.[5,6] Furthermore, by placement of N-terminal groups bearing heterocyclic rings on oligopeptide chains, subsequent transformations, such as Diels−Alder reactions, can be performed to produce oxo-norbornene macromonomers that, in turn, can be used for polymerizations to prepare polymers with controlled architecture.[7−10]

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

Conventional synthesis of end-functionalized peptides discussed above involves either solid phase or liquid phase peptide synthesis.[4,7,10−12] These synthetic methods provide peptides in high purity with a precise sequence and chain length. However, both solid phase and liquid phase peptide synthesis is costly, uses toxic reagents, and requires protection−deprotection chemistry and product purification. Fermentative routes to peptides are also important but generally suffer from low product yields and are inherently difficult to adapt toward incorporation of non-peptidic end-group moieties.[12−16]

To increase the viability of using peptides in an ever-expanding range of exciting applications, new methods for peptide synthesis are needed that are safe, scalable, and cost-effective. When a single product with a precise amino acid sequence is not required, there is an opportunity to introduce new peptide synthetic methodologies that are simple, cost-effective, and environmentally friendly. For this purpose, our laboratory is developing protease-catalyzed routes to peptides from amino acid alkyl esters in buffered aqueous media.

The following provides representative literature documenting important progress toward protease-catalyzed oligopeptide synthesis. Protease-catalyzed oligomerization of dialkyl L-glutamate hydrochloride was reported by Aso et al.[17,18] Papain catalysis in a buffer of methionine, phenylalanine, threonine, and tyrosine ester hydrochlorides gave poly([α]-amino acids) with an average degree of polymerization (DP_{avg}) less than 10.[19−21] To determine regioselectivity of oligoglutamate oligomers, $^1H-^1H$ COSY NMR were recorded and analyzed.[19,20] It was concluded that oligomers formed consist exclusively of α-linked γ-ethyl glutamate units. Li et al.[22] assessed the influence of reaction conditions on papain-catalyzed

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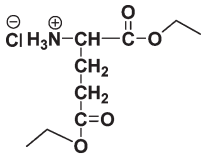
γ-L-$(Et)_2$-Glu oligomerizations. Yields of ~80% oligo(γ-L-Et-Glu) were obtained in 15 min.[22] Li et al.[23] also studied co-oligomerizations of L-leucine ethyl ester (L-Et-Leu) and Et_2-L-Glu. The relative activity of protease catalysts for this co-oligomerization was as follows: papain≈bromelain > 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 L-Leu terminal unit.[23]

Protease catalysis has been used in two-step reactions to conjugate a specific amino acid or peptide sequence to a preformed peptide synthesized by solid phase or liquid phase peptide synthesis. Also, work has explored the promiscuity of protease-catalyzed coupling reactions using either non-natural amine nucleophiles or acyl donors. For example, a β-peptidyl aminopeptidase was used to catalyze bond formation between β-amino acids and free N-termini of α-tripeptides.[24,25] Investigations were performed to determine the efficiency of cysteine and serine protease-catalyzed coupling of N-protected amino acids and peptides to 4-aminoantipyrine.[26] Schuster et al.[27] investigated protease-catalyzed coupling reactions under frozen conditions using N-Mal-Tyr-OMe (Mal = maleyl) as the acyl donor species. Non-natural amine nucleophiles were studied for concurrent digestion and C-terminal functionalization of peptides using trypsin, α-chymotrypsin, and elastase as catalysts.[28] Gunther et al.[29] explored the tolerance of Clostripain, also known as endoproteinase Arg-C, for coupling reactions using a series of non-natural amino acid acyl donors activated by the formation of their 4-guanidinophenylester derivatives. Also, carboxypeptidase Y was active for catalysis of amide bond formation using reduced amino acid nucleophiles L-phenylalaninol and L-histadinol.[30]

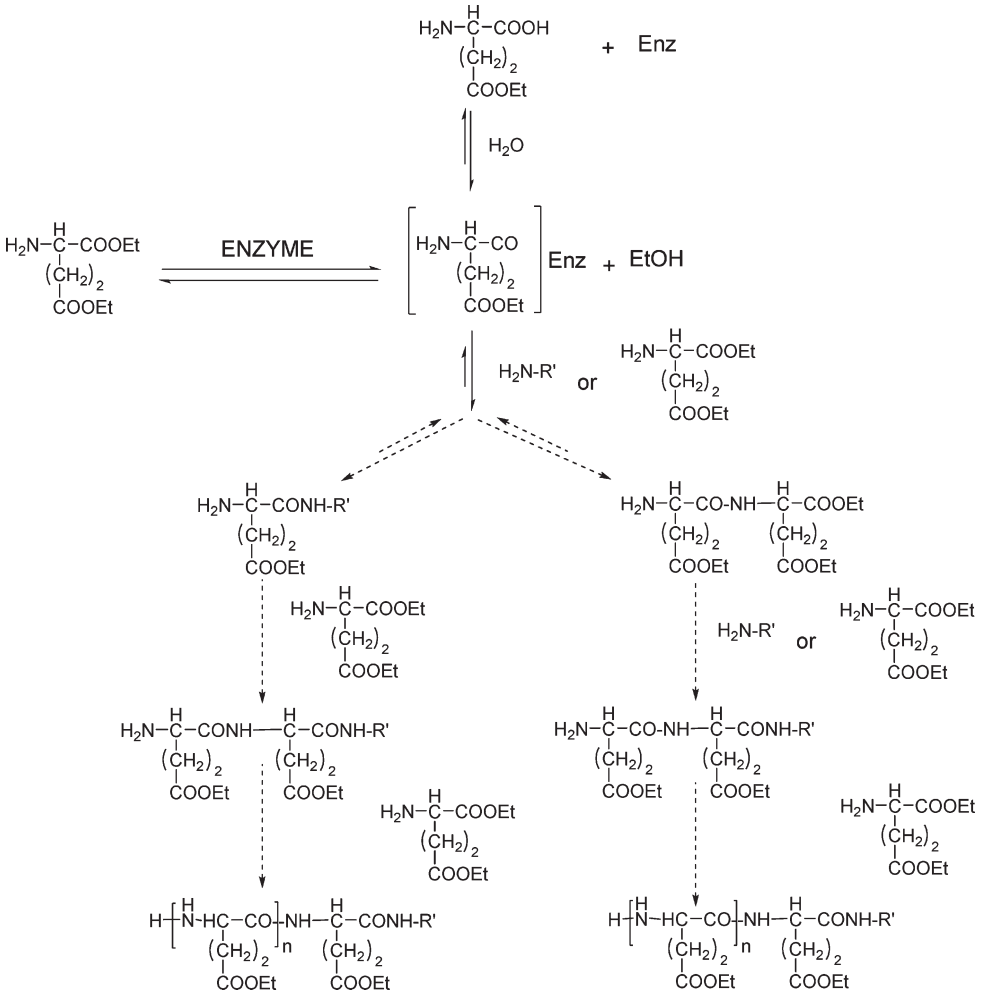
Scheme 1. Structure of Diethyl Glutamic Acid Hydrochloride Substrate Used in Protease-Catalyzed Oligomerization and It's In Situ C-Terminal End for End-Functionalization



γ-L-$(Et)_2$-Glu.HCl

Literature examples above affirm the importance of end-functionalized peptides for a variety of applications in material science and the need to develop simple methods for their preparation. Hence, in this paper, we report a unique and facile one-pot biotransformation for the preparation of oligo(γ-L-Et-Glu) decorated at the C-terminus with selected amine-functionalized end-groups (see Scheme 2). Papain, bromelain, α-chymotrypsin, Multifect P-3000, and Purafect prime 4000 L were used as catalysts for oligomerization of γ-L-$(Et)_2$-Glu in the presence of mono functional amines. For each protease, preferred pH values for conversion of γ-L-$(Et)_2$-Glu to oligo(L-γ-Et-Glu) were first determined. Non-natural amine nucleophiles with subtle differences in structure were interrogated to derive insights into protease promiscuity to

Scheme 2. Proposed Model of Concurrent Peptide Synthesis and In Situ End-Capping of the Oligopeptides



prepare C-terminal functionalized oligo(L-γ-Et-Glu). The amine nucleophiles or acyl acceptors studied each mimic phenylalanine in that they have aromatic rings linked to amine groups by one or more methylenes. Reactions were performed using the above proteases at their preferred pH with variation in the ratio of monomer γ-L-$(Et)_2$-Glu to amine phenylalanine analog. ^{1}H NMR was used to identify the presence and mol-% of C-terminal units. Further confirmation of NMR results was obtained by MALDI-TOF analysis. The cumulative results of this paper demonstrate a general method that is simple and scalable by which oligopeptides can be prepared from protease catalysis from one or more amino acid alkyl esters in one-pot reactions with control of end-group structure.

Experimental Section

Materials. L-Diethyl glutamic acid hydrochloride [L-$(Et)_2$-Glu·HCl] was purchased from Tokyo Kasei Co. Ltd., and 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. Water insoluble materials in the as-received papain were removed by dissolving 300 mg/mL crude papain powder in deionized water, centrifugation at 5000 rpm for 30 min, collecting the clear supernatant, and discarding the insoluble precipitate. The clear supernatant was lyophilized overnight to obtain fully water-soluble papain as a beige powder that was used for all studies herein. Bromelain (cysteine protease; EC 3.4.22.4; source *pineapple stem*; 3.4.22.32, protein content ≥ 35% protein by biuret, 1.7 units/mg protein), α-chymotrypsin type II (serine protease; EC 3.4.21.1; from bovine pancreas, 83.9 units/mg, 96 units/mg protein), protease SG (serine protease), trypsin (serine protease; EC 3.4.21.4; source bovine pancreas; ≥10000 BAEE units/mg protein), protease subtilisin Carlsberg type VIII (serine protease, EC 3.4.21.14; source *Bacillus licheniformis*; 7−15 units/mg solid by casein assay), protease Sg (serine protease; source, *Streptomyces griseus*; ≥4 units/mg solid by casein assay), proteinase type XXVII (EC 3.5.1.14; source *Aspergillus melleus*; ≥3 units/mg of solid by casein assay) were purchased from Sigma Aldrich. Multifect P-3000 (serine protease; IUB 3.4.21.62; from a genetically modified strain of *Bacillus subtilis*; 2750 GSU (Genencor subtilisin units/g), Purafect prime 4000 L (serine protease; IUB 3.4.21.62; from *Bacillus amyloliquefaciens*; 4000 PPU (purafect prime units)/g), and alkaline protease (serine protease) were kind gifts from Genencor International. L-Phenylalaninol and L-histidinol were purchased from Alfa Aesar. Bicyclo[2.2.1]-5-heptene-2-carbonitrile was purchased from Frinton laboratories. 2-Thiophene methyl amine, 2-thiophene ethyl amine, 2-furfurylamine, 5-methyl-2-furfurylamine, benzylamine, 2-methylamino pyridine sodium phosphate diabasic, sodium acetate, casein, deuterated dimethyl sulfoxide (DMSO-d_6), Folin and Ciocalteu's reagent, trichloroacetic acid, and α-cyano hydroxycinnamic acid (CCA, MALDI-TOF matrix) were all purchased from Aldrich. Deionized water (DI, 18.2 MΩ·cm purity) was obtained from a RIOS 16/MILLQ Synthesis Millipore water purification system. 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*). The following procedure follows that described in a technical bulletin for a protease colorimetric detection kit (product code PC0100 [Sigma Aldrich]). In summary, a dilute solution of the respective enzyme (25 μL) was transferred to a 1 mL microfuge tube. Casein solution (130 μL, 0.65%) was then added to the microcentrifuge tube and the solution was incubated at 37 °C for 10 min. Then, a solution of trichloroacetic acid (TCA, 130 μL, 110 mM) was added and the combined solution was further incubated for 20 min. Subsequently, the solution was centrifuged and 250 μL of supernatant was assayed by addition into a Na_2CO_3 solution (625 μL, 500 mM), with Folin and Ciocalteu's phenol reagent (125 μL). The absorption was measured at 660 nm. Activity was calculated as follows:

$$\text{units/mL enzyme} = \frac{(\mu\text{mol tyrosine equivalents released})(A)}{(B)(C)(D)}$$

where A = total volume (mL) of assay; B = time of assay (min) as per the unit definition; C = volume of enzyme used (mL); and D = total volume (mL) used in colorometric determination.

Tyrosine equivalents in μmol released is determined by using the equation constructed from the standard graph for L-tyrosine (absorption vs concentration).

General Procedure for Protease-Catalyzed Oligo(γ-L-Et-Glu) Synthesis. The method for oligo(γ-Et-L-Glu) synthesis was adapted from a literature procedure.[22] In summary, L-$(Et)_2$-Glu·HCl (600 mg, 2.5 mmol), protease (16 units/mL), and 5 mL of 0.9 M sodium phosphate buffer solution set at a predetermined pH (commensurate with the optimum pH for oligo(γ-L-Et-Glu) synthesis for the protease used) 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 precipitate.

Synthesis and Characterization of C-Terminus Modified Oligo(γ-L-Et-Glu). The method used is identical to that for synthesis of oligo(γ-Et-L-Glu) except for the following modifications. Although the stoichiometry of L-$(Et)_2$-Glu·HCl and end-capping substrate was varied, the total concentration of monomer and end-capping substrate remained at 2.5 mmol. Due to the high basicity/acidity of end-capping nucleophiles (NH_2-R), sodium phosphate buffer solution (5 mL, 0.9M) was added as above and solutions were titrated back to the desired pH using 10 M NaOH or 10 M HCl. Subsequently, 16 units/mL of the desired enzyme was added to catalyze oligopeptide synthesis. Precipitate formed was washed with 2 × 5 mL of deionized water then with an HCl solution (pH 2, 2 × 5 mL), separation of precipitate after each washing step was by centrifugation (5000 rpm), supernatants were discarded, and the precipitate was freeze-dried to obtain the product. The % yield of amine end-capped chains of oligo(γ-L-Et-Glu) [oligo(γ-L-Et-Glu)-NH-R] was calculated gravimetrically from the precipitated product obtained.

^{1}H NMR chemical shifts (δ in ppm) in DMSO-d_6 of oligo(γ-L-Et-Glu): 1.16 (t, −CH_3), 1.84 (d, −CH_2), 2.30 (m, −CH_2), 3.78 (bs, −CH), 4.03 (m, −CH_2), 4.25 (bs, −CH), 7.8−8.6 corresponding to (−CONH) region of oligo(γ-Et-L-Glu).

^{1}H NMR chemical shifts (δ in ppm) in DMSO-d_6 of end-functional groups linked to oligo(γ-L-Et-Glu: 2-amino methylfuran (FMA): 6.2 ([(d, −CH], furan ring 3-position), 6.38 ([d, −CH], furan ring 4-position), 7.5 ([d, −CH], furan ring 5-position); 2-thiophene ethylamine (TPEA): 2.91 ([t, 2H, −CH_2] of *TPEA*), 6.9 ([bd, 2H, −CH], thiophene ring 1,3-positions), 7.3 ([bs, −CH], thiophene ring 4-position); 5-methyl-2-amino methylfuran (MFMA): 2.2 ([bs, −CH_3], substituted at furan ring 5-position), 5.9 ([bd, −CH], furan ring 4-position), 6.08 ([bd, −CH], furan ring 3-position); 4-methylamino pyridine (MPy): 4.07 ([m, −CH_2], substituted at pyridine ring 4-position and [−CH_2] region of the oligo[γ-L-Et-Glu] ester group), 7.22 ([m, 2H,-CH], pyridine ring 3,5 position), 7.8−8.6 ([m, 2H, −CH], pyridine ring 1,6 positions and [−CONH] region of oligo[γ-Et-L-Glu]); benzylamine (*BzA*): 4.1 (m, −CH_2, substituted at benzene ring 1-position and −CH_2 region of oligo[γ-Et-L-Glu] ester group, 7.28 (m, 5H, [−CH], benzene ring positions 2−6).

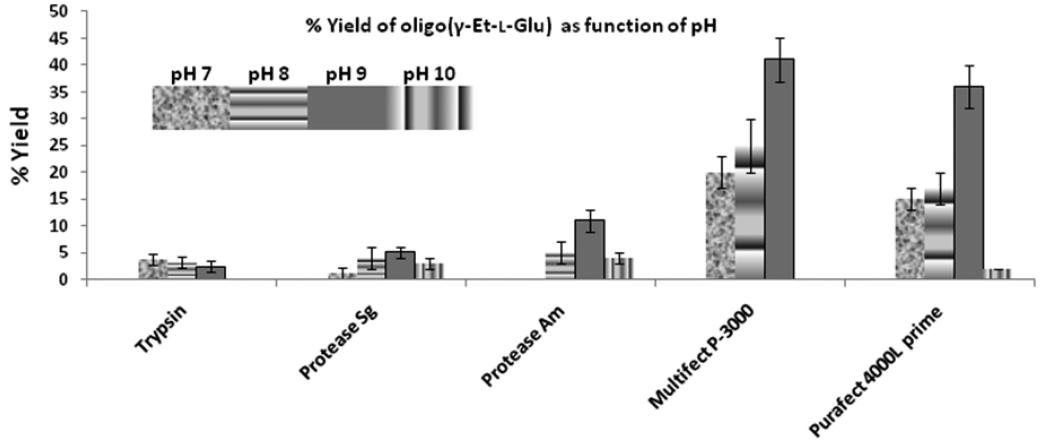


Figure 1. Evaluation of proteases for conversion of γ-L-$(Et)_2$-Glu to oligo(γ-L-Et-Glu) as a function of medium pH. Error bars represent the deviation from the mean of duplicate experiments.

Instrumental Methods

Nuclear Magnetic Resonance (*NMR*). Proton (^{1}H) NMR spectra were recorded on a Bruker DPX 300 spectrometer at 300 MHz. Products (10 mg/mL) were dissolved in deuterated dimethyl sulfoxide (DMSO-d_6) and a total of 128 scans were collected and analyzed by MestRec-C 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 was used for hardware control and calibration. Spectra were obtained by averaging at least 300 laser shots. The pulsed ion extraction delay time was 200 ns. The spectrometer was calibrated using Angiotensin II as the external standard (1046.54 amu). To generate the matrix, a saturated solution of α-cyano-4-hydroxycinnamic acid (CCA) was prepared in a water/acetonitrile (2:1 v/v) with 0.1% TFA (TA solution). Oligopeptide samples dissolved in 10 μL DMSO with 0.1% TFA were diluted with 240 μL of TA solution so that the final concentration of oligopeptide was ~10 pmol/μL. A 5 μL aliquot of this solution was mixed with 5 μL of CCA (matrix) solution in a 100 μL eppendorf tube. Then, 0.5 μL of this mixture was applied to the steel target that was then dried in ambient air. The abundance intensities of peaks at m/z values were collected via X-massOMNIFLEX6.0.0. software. Molecular weights obtained by experimental data were compared to a theoretical database created in MS Excel for the different end-capped peptides.

Results and Discussion

Conversions of γ-L-$(Et)_2$-Glu to oligo(γ-L-Et-Glu) as well as C-terminal functionalization is driven kinetically by using the activated Glu-α-ethyl ester.[19,31,32] Reactions are also thermodynamically driven toward oligomer formation by product precipitation when chain lengths reach $DP_{avg} \sim 8$.[22,23,33] Hence, competing reactions due to hydrolysis in the aqueous reaction media are largely overcome. Scheme 2 shows these characteristics as well as the devised one-pot reaction to form C-terminal functionalized oligopeptide. It is apparent that potential pathways to products can occur by (*i*) reaction between NH_2-R and γ-L-Et_2-Glu to form γ-L-Et-Glu-NH-R that subsequently propagates to form oligomeric product or (*ii*) oligomerization of γ-L-$(Et)_2$-Glu to oligo(γ-L-Et-Glu)$_n$ followed by reaction with NH_2-R to form oligo(γ-L-Et-Glu)$_n$-N*H*-*R*. The latter can precipitate or continue to propagate adding x γ-L-$(Et)_2$-Glu units prior to precipitation (forming oligo(γ-L-Et-Glu)$_{n+x}$-NH-R).

Protease Characterization. All proteases used herein were characterized to determine their protein content by the BCA method[34,35] and hydrolytic activity by a casein assay (see Experimental Section). Values obtained from these measurements are given in the Supporting Information section (Figures S1 and S2). The amount of protein used in reactions was normalized based on casein hydrolysis activity. In summary, the protease samples, Multifect P-3000, Purafect prime 4000 L, and alkaline protease had low protein content but very high (casein) hydrolytic activity. In contrast, α-chymotrypsin, protease Sg, and trypsin had high protein content and high hydrolytic activity, while papain, bromelain, and proteinase Am had low protein content and moderate hydrolytic activity. In all studies that follow, 16 units/mL (a unit is the quantity of protease required to release 1 μmol of tyrosine equivalents per min per mL at pH 7.5 and 37 °C) of protease was used to catalyze oligo(γ-L-Et-Glu) synthesis and concurrent C-terminal functionalization with amine nucleophiles [oligo(γ-L-Et-Glu)-NH-R].

Optimum pH for Oligopeptide Synthesis. Li et al.[23] reported that optimal pH values for conversion of γ-L-$(Et)_2$-Glu to oligo(γ-L-Et-Glu) by papain, bromelain, α-chymotrypsin, and protease Sg are 6−8, 8−9, 9, and 8−9, respectively. Because % yields of oligo(γ-L-Et-Glu) using papain, bromelain, and α-chymotrypsin exceeded 30%, these three proteases were selected for studies herein on concurrent oligo(γ-L-Et-Glu) synthesis and end-capping reactions.[22,23] Along with the above enzymes, additional proteases were evaluated for use. Trypsin, protease Sg, protease Am, Multifect P-3000, and Purafect 4000 L prime were assayed for oligo(γ-L-Et-Glu) synthesis at initial pH values of 7, 8, 9, and 10 (see Figure 1). Only Multifect P-3000 and Purafect prime 4000 L gave oligo(γ-L-Et-Glu) in yields >30%. Thus, in addition to papain, bromelain, and α-chymotrypsin, Multifect P-3000 and Purafect prime 4000 L were selected for C-terminal modification studies. Trypsin, protease Sg, and protease Am gave oligo(γ-L-Et-Glu) yields up to 11% at initial pH = 9. Thermolysin, protease AO, protease T, and Purafect 4000 L gave no precipitate, suggesting that all these proteases were inactive for γ-L-$(Et)_2$-Glu oligomerization to chain lengths with $DP_{avg} \sim 7$. For both Multifect P-3000 and Purafect prime 4000 L, an increase in initial pH from 8 to 9 gave corresponding increases in product yield (25 to 41% and 17 to 36%, respectively).

With respect to hydrolytic activities, optimal pH values for trypsin, protease Sg, protease Am, Multifect P-3000, and Purafect prime 4000 L occurs at 7−9, 5−9, 6−8, 7.5, and 6.5−10.5, respectively. In contrast, pH optima for oligo(γ-L-Et-Glu)

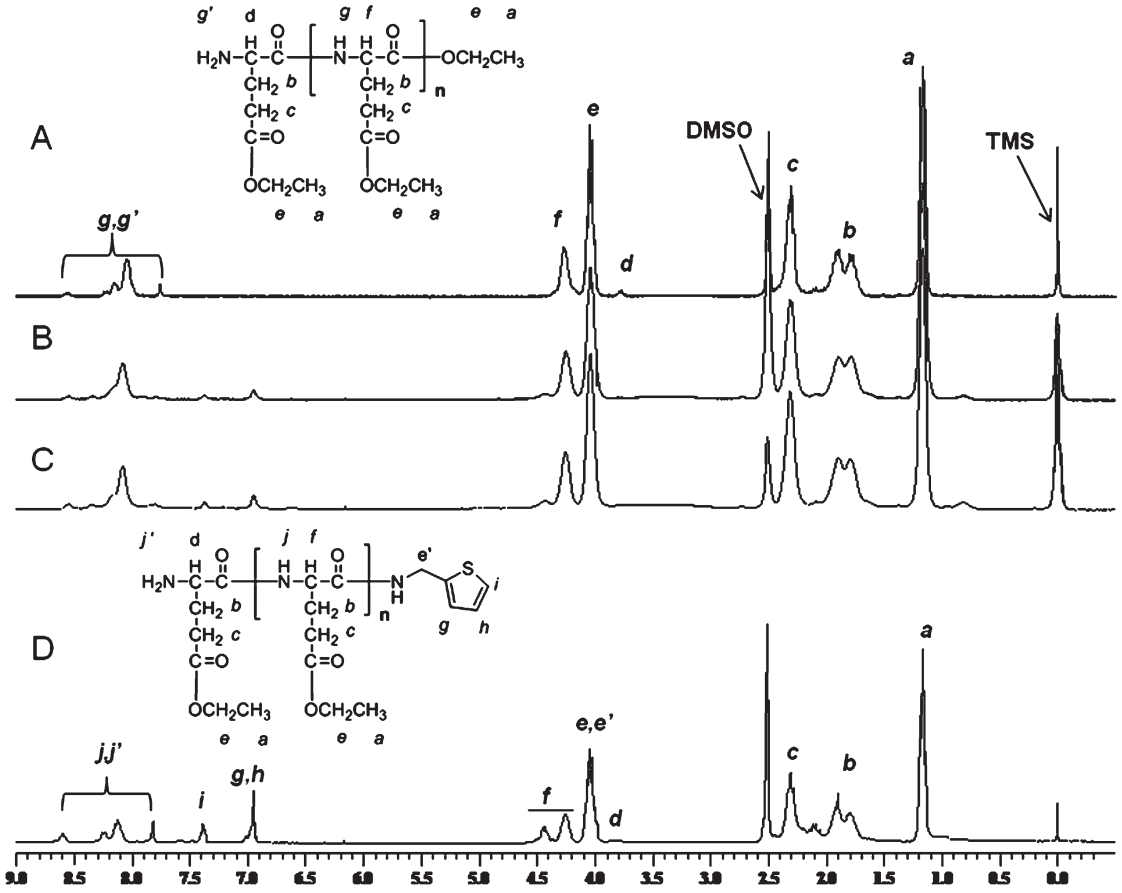


Figure 2. ^{1}H NMR (300 MHz, DMSO-d_6) spectra of oligopeptides synthesized with total monomer concentration of 0.5 M in 0.9 M phosphate buffer at 40 °C for 3 h: (A) oligo(γ-L-Et-Glu) synthesized using Multifect P-3000 as catalyst; (B) TPMA end-capped oligo(γ-L-Et-Glu) using papain as catalyst and feed ratio of 7:3 γ-L-Et$_2$-Glu-to-TPMA; (C) TPMA end-capped oligo(γ-L-Et-Glu) using bromelain as catalyst and feed ratio of 7:3 γ-L-Et$_2$-Glu-to-TPMA; (D) TPMA end-capped oligo(γ-L-Et-Glu) using Multifect P-3000 as catalyst and feed ratio of 7:3 γ-L-Et$_2$-Glu-to-TPMA.

synthesis with these proteases occurs at 7, 9, 9, 9, and 9, respectively.[23] Similarly, papain, bromelain, and α-chymotrypsin have optimum hydrolytic activity at pH 6−8, 4−8, 6−9, respectively, and optimum pH values for synthetic activity at 8, 8, and 9, respectively. Hence, with the exception of trypsin, high pH values within or above the range of protease activity for peptide bond hydrolysis were preferred for peptide synthesis.[22,23,36,37]

These results are explained by considering that α-NH_2 moieties of amino acids have pK_a values of 8.1−10.6. For γ-L-(Et)$_2$-Glu, the α-NH_2 pK_a is ∼9.4. Therefore, by shifting the pH to values close to or above 9, an increase in the population of free or nonprotonated NH_2 groups occurs, thereby increasing their reactivity for accepting alkyl-activated acyl groups. Accordingly, by the same principle, reactions between amine nucleophiles NH_2-R with γ-L-(Et)$_2$-Glu or propagating γ-L-Et-Glu oligomers should be enhanced at high pH values that favor a larger population of nucleophiles in the free amine form. Nevertheless, the reaction pH cannot be increased to a value that would result in protease denaturation, thereby eliminating the three-dimensional protein structure that supports protease activity.

Overall, based on oligo(γ-L-Et-Glu) yields at optimal pH values for this synthetic reaction, the relative order of protease activities is as follows: papain∼bromelain > α-chymotrypsin > Multifect P-3000 > Purafect prime 4000 L > protease Sc ∼ proteinase Am∼protease Sg∼trypsin. Other proteases investigated appeared inactive for conversion of γ-L-(Et)$_2$-Glu to oligo(γ-L-Et-Glu) synthesis. The DP_{avg} of oligo(γ-L-Et-Glu) synthesized by these proteases was determined by ^{1}H NMR (see discussion below). Oligo(γ-L-Et-Glu) DP_{avg} values were one or two units shorter for oligomers prepared by trypsin, Protease Sg and Proteinase Am (8.5, 8.3, 8.2, respectively) relative to those prepared by Multifect P-3000 and Purafect 4000 L prime (9.2 and 9.5, respectively).

Structural Analysis by ^{1}H NMR Spectroscopy. The spectrum for Multifect P-3000 catalyzed oligo(γ-L-Et-Glu) is shown in Figure 2A. Peak assignments were based on published literature.[19,22] Methine resonances of γ-L-Et-Glu repeat units (including those of C-terminal resonances) are found at 4.25 ppm (protons *f*), whereas N-terminal methine resonances (protons *d*) for γ-L-Et-Glu units are at ∼3.7 ppm. Hence, a direct comparison of signal intensities at 4.3 and 3.7 ppm was used to determine DP_{avg} values of oligo(γ-L-Et-Glu). For Multifect P-3000 catalyzed reactions of TPMA with γ-L-(Et)$_2$-Glu, a shoulder with peaks in the region 4.3−4.45 ppm appeared corresponding to protons of C-terminal modified γ-L-(Et)-Glu repeat units. Relative integration of methine proton signals *f* at 4.25−4.45 and *d* at 3.7 ppm, respectively, was used to calculate DP_{avg}. Unless otherwise specified in the Experimental Section, protons *f* remain at ∼4.25−4.45 ppm when other nucleophiles in Scheme 1 replace TPMA at the C-terminal site of oligo(γ-L-Et-Glu). The percent C-terminal modification by reaction with NH_2-R nucleophiles in Scheme 1 was determined from the relative integration of protons corresponding to NH_2-R and the side chain protons of *b* corresponding to α-CH_2 of oligo(γ-L-Et-Glu).

Following the above-mentioned protocol, the percent C-terminal modification by reaction with NH_2-R nucleophiles in Scheme 1 was determined from relative intensities of

Table 1. Percent Yield and Oligo(γ-L-Et-Glu) Modified at the C-Terminus with NH_2-R End-Capping Nucleophiles as a Function of the γ-L-Et_2-Glu to R-NH_2 Ratio, Enzyme Origin, and NH_2-R Structure[a,b]

NH_2-R nucleophile	enzyme	γ-L-Et_2-Glu to NH_2-R nucleophile[c] 7:3[c]	5:5	3:7	7:3	5:5	3:7
		% yield[d]			% modified chains[d]		
TPMA	papain	63	40	20	25	50	70
	bromelain	49	20	10	50	60	>90
	α-chymotrypsin	18	10	5	75	0	0
	Multifect P-3000	31	0	0	>90	0	0
	Purafect prime 4000 L	17	0	0	>90	0	0
TPEA	papain	63	50	10	20	25	0
	bromelain	33	15	0	0	0	0
	α-chymotrypsin	23	10	0	0	0	0
	Multifect P-3000	5	0	0	0	0	0
	Purafect prime 4000 L	5	0	0	0	0	0
FMA	papain	50	45	15	15	20	30
	bromelain	45	40	10	25	40	45
	α-chymotrypsin	30	15	3	37	50	0
	Multifect P-3000	20	0	0	42	0	0
	Purafect prime 4000 L	3	0	0	50	0	0
MFMA	papain	47	27	0	20	44	0
	bromelain	36	0	0	22	0	0
	α-chymotrypsin	22	10	0	36	50	0
	Multifect P-3000	0	0	0	0	0	0
	Purafect prime 4000 L	0	0	0	0	0	0

[a] Reactions were carried out in 0.9 M phosphate buffer, for 3 h, at 40 °C and at the pH optimum for each enzyme used. [b] Yield % error was less than ±6%. [c] Underlined number gives the value in the ratio of NH_2-R. [d] Percent yield and modified chains was determined assuming oligopeptides consist of eight γ-Et-Glu repeat units. For products containing C-terminal modified moieties [oligo(γ-L-Et-Glu)-NH-R], the % yield was calculated by subtracting the weight contributed by C-terminal moieties as determined by ^{1}H NMR. Thus, % yield is total product yield [oligo(γ-L-Et-Glu) + oligo(γ-L-Et-Glu)-NH-R], where the contribution of NH_2-R to product weight is subtracted.

oligo(γ-Et-L-Glu) α-CH_2 side chain protons *b* and protons *i*, *h*, and *g* between 6.9 and 7.5 ppm of the TPMA thiophene moiety. Assignments for ^{1}H NMR signals that were used to quantify other R-NH_2 groups used in this study are listed in the Experimental Section. ^{1}H NMR spectra in Figure 2B−D show how varying the protease used (papain, bromelain, and Multifect P-3000, respectively) results in changes in efficiency of end-capping reactions. Reactions investigated used TPMA as the amine nucleophile and the ratio of TPMA-to-γ-L-Et_2-Glu in the monomer feed was maintained at 7:3. The discussion below relies upon ^{1}H NMR as above to quantify the extent that different NH_2-R nucleophiles react with ester groups (-[C═O]-O-Et) of either propagating oligo(γ-L-Et-Glu) chains or monomer (γ-L-Et_2-Glu).

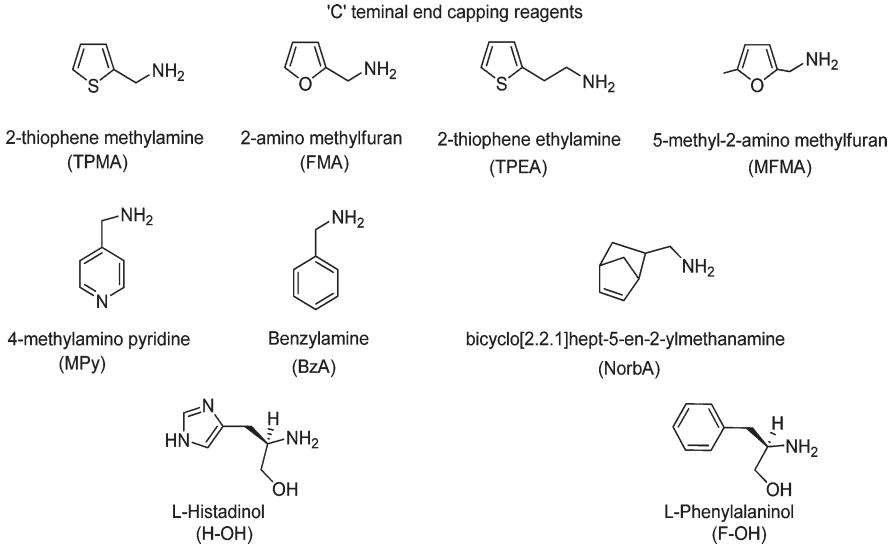
Table 1 lists cumulative % yields of precipitated oligo(γ-L-Et-Glu) chains with and without C-terminal end-capped oligo(γ-L-Et-Glu) chains as well as the percent of oligo(γ-L-Et-Glu) chains modified at the C-terminus with NH_2-R end-capping nucleophiles. The variables studied included the protease origin, ratio of γ-L-Et_2-Glu-to-NH_2-R in the monomer feed and NH_2-R structure. Reactions were performed in 0.9 M sodium phosphate buffer solution at 40 °C for 3 h. Initial medium pH was adjusted to 8, 8, 9, 9, and 9, corresponding to pH optima values determined above for papain, bromelain, α-chymotrypsin, Multifect P3000, and Purafect prime 4000 L, respectively. Without amine nucleophile in reactions, oligo(γ-L-Et-Glu) % yield with the above proteases is 75 ± 4%, 76 ± 3 and 45 ± 5%, and 41 ± 4 and 36 ± 4%, respectively. Inspection of Table 1 shows that, for all five proteases and amine nucleophiles studied, addition of increased quantities of amine nucleophiles from 0 to 30, 50, and 70 mol % with respect to γ-L-Et_2-Glu results in decreased % yield but increased mol % of [oligo(γ-L-Et-Glu)-NH-R]. These trends were intuitively expected because an increase in the ratio of NH_2-R/γ-L-Et_2-Glu groups (*i*) can cause an increase in the number of chains formed, thereby creating a fraction of reduced DP_{avg} oligo(γ-L-Et-Glu)-NH-R that does not precipitate, (*ii*) results in a reduced concentration of oligomerizable substrate in reactions, and (*iii*) NH_2-R might also function as a competitive inhibitor.

Of the different NH_2-R compounds evaluated, TPMA gave the highest fraction of oligo(γ-L-Et-Glu)-NH-R chains in the precipitated product. Intriguingly, this result is true regardless of the protease used. However, distinct differences were found in the capability of different proteases to form oligo(γ-L-Et-Glu)-TPMA. For instance, with γ-L-Et_2-Glu to TPMA of 7:3, the mol % of oligo(γ-L-Et-Glu)-TPMA formed with papain, bromelain, α-chymotrypsin, Multifect P-3000, and Purafect prime 4000 L is 25, 50, 75, >95, and >95, respectively. Also, using bromelain catalysis and ratios of Et_2-Glu to TPMA of 5:5 and 3:7, the mol % of oligo(γ-L-Et-Glu)-TMPA formed were 80 and >90%, respectively. For Multifect P-3000, the attainment of >95 mol % of chains with TPMA C-terminal groups is particularly exciting because the decrease in product yield relative to 0 mol % TPMA in reactions was only 10% (41 to 31%).

Given the high reactivity of TPMA for protease-catalyzed C-terminal end-capping, work was performed to explore structural analogs of TPMA. The goal was to derive insights into protease promiscuity and, thereby, an understanding of the range of related amine nucleophiles that may be incorporated at oligo(γ-L-Et-Glu) C-termini. TPMA and TPEA differ structurally by one methylene unit between the thiophene and amine groups (see Chart 1). Table 1 shows that, of the five proteases studied, only papain formed oligo(γ-L-Et-Glu)-TPEA chains. For example, the reaction catalyzed by papain with γ-L-Et_2-Glu-to-TPEA 5:5 mol/mol, 25 mol % of precipitated product consisted of oligo(γ-L-Et-Glu)-TPEA. Multifect P-3000 and Purafect prime 4000 L, which gave >90 mol % of oligo(γ-Et-Glu)-TPMA with γ-L-Et_2-Glu to TPEA 7:3, was ineffective in forming oligo(γ-L-Et-Glu)-TPEA. This large difference in reactivity between TPMA and TPEA, found for all five proteases studied, can be explained by that TPMA more closely resembles α-amino acids.

A close analog of TPMA is FMA, which contains a furan in place of the thiophene ring. Comparison of total product

Chart 1. Structures and Abbreviations of Primary Amine Nucleophiles (NH_2-R) Used in this Study for End-Functionalization of Oligo(γ-L-Et-Glu)



yield [oligo(γ-L-Et-Glu) + oligo(γ-L-Et-Glu)-FMA] obtained as a function of the γ-L-Et_2-Glu-to-FMA ratio in the monomer feed was generally similar. However, for all the proteases used, the mol % of oligo(γ-L-Et-Glu)-FMA chains was clearly less than what was obtained using TPMA as NH_2-R. For example, with Et_2-Glu to FMA of 7:3, the mol % oligo(γ- L-Et-Glu)-FMA formed with papain, bromelain, α-chymotrypsin, Multifect P-3000, and Purafect prime 4000 L is 15, 25, 37, 42, and 50%, respectively. In comparison, using TPMA, the mol % of oligo(γ-L-Et-Glu)-TPMA formed was 25, 50, 75, >90, and >90, respectively. Therefore, substitution of thiophene with a furan ring had a large effect on NH_2-R efficiency.

FMA and MFMA differ by a methyl substituent at the furan ring 5-position. Comparison of total product yield [oligo(γ-L-Et-Glu) + oligo(γ-L-Et-Glu)-MFMA] obtained as a function of γ-L-Et_2-Glu to MFMA showed that, for all five proteases, yields were lower when using MFMA instead of FMA as NH_2-R. For example, for γ-L-Et_2-Glu to NH_2-R 5:5 and using bromelain as catalyst, product yield was 40 and 0% with FMA and MFMA, respectively. When using α-chymotrypsin, Multifect P-3000, and Purafect prime 4000 L, mol % incorporation of FMA and MFMA at C-termini were similar. In contrast, with papain as catalyst and γ-L-Et_2-Glu to NH_2-R of 5:5, incorporation of MFMA was higher than FMA (44 vs 20%). As discussed above, lower product yields obtained with MFMA may be due to an increase in the number of chains formed resulting in a fraction of oligo(γ-L-Et-Glu)-NH-R that does not precipitate or to its function as a competitive inhibitor.

As was observed in Table 1, Table 2 results show that, for all five proteases and amine nucleophiles studied, addition of increased quantities of amine nucleophiles from 0 to 30, 50, and 70 mol % with respect to Et_2-Glu resulted in decreased % yield of precipitated product but increased contents of oligo(γ-L-Et-Glu)-NH-R. Also, the extent of decreased yield and increased end-capping was highly sensitive to the protease-NH_2-R pair studied. Of interest was to explore amino acids as C-terminal end-capping compounds, where the acid moiety is nonactivated by esterification (e.g., in free acid or salt form).[30,38] Given its importance in metal binding, L-histidine (*H*) was studied as an amine nucleophile during oligo(γ-L-Et-Glu) synthesis. Using papain, bromelain, and α-chymotrypsin as protease catalysts and a feed ratio of 5:5 γ-L-Et_2-Glu/*H*, total % product yields obtained were 40, 45, and 15%, respectively. ^{1}H NMR and MALDI-TOF spectra for L-histidine end-capping experiments showed an absence of peaks corresponding to incorporation of *H* at oligo(γ-L-Et-Glu) C-termini. Furthermore, when *H* was employed as amine nucleophile using Multifect P-3000 and Purafect prime 4000 L as catalysts, no precipitate was obtained at all feed ratios studied. Furthermore, for all five proteases studied herein, L-phenylalanine (*F*) addition to γ-L-Et_2-Glu did not produce end-capped oligo(γ-L-Et-Glu)-*F*. Interestingly, previous attempts documented in the literature to use inactivated *F* and *H* as amine nucleophiles gave low product yields. Specifically, carboxypeptidase-Y catalyzed reaction of Bz-Ala-(C=O)-OMe with *F* and *H* gave dipeptide in 25 and 15% yield, respectively. Furthermore, thermolysin-catalyzed coupling of H_2N-(O=C)-His-NH_2 (α-carboxyl amide derivative of *H*) with Z-Phe-OH resulted in no dipeptide formation.[39]

In an effort to understand what structural features of these natural amino acids resulted in their inability to react as amine nucleophiles during oligo(γ-L-Et-Glu) synthesis, further studies were performed with both *F* and *H* analogs. L-Phenylmethanamine (BzA) is similar to *F* but lacks an α-carboxyl group. For γ-L-Et_2-Glu to BzA 7:3, the mol % of oligo(γ-L-Et-Glu)-B*zA* formed with papain, bromelain, and α-chymotrypsin is 20, 36, and 30%, respectively. When using Multifect P-3000 and Purafect prime 4000 L as catalysts, no precipitated product was formed. By increasing the molar ratio of γ-L-Et_2-Glu-to-BzA to 3:7, the mol % of oligo(γ-L-Et-Glu)-BzA with papain and bromelain increased to 55 and 62%, respectively. Thus, relative to TPMA, BzA was not as effective in forming end-group modified products. However, substitution of the α-carboxyl group of *F* with a hydrogen atom was found to be beneficial to enabling BzA to function as a C-terminal end-capping group during synthesis of oligo-(γ-L-Et-Glu).

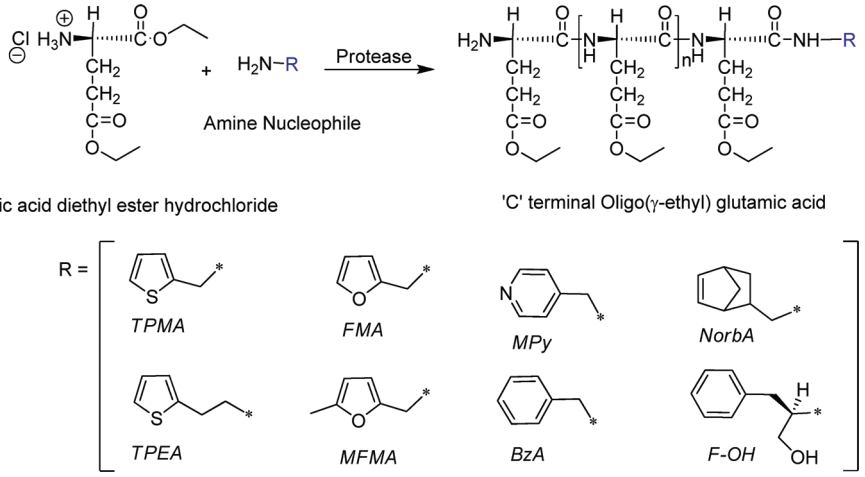
A close analog of BzA is 4-methylamino pyridine (MPy), which contains a pyridine ring in place of the phenyl ring. Comparison of total product yield obtained as a function of γ-L-Et_2-Glu to MPy was generally similar. The relative efficiency of BzA and MPy end-capping of oligo(γ-L-Et-Glu) chains during oligomer synthesis changed as a function of the protease used. For papain and bromelain, incorporation of BzA was higher than MPy. However, for γ-L-Et_2-Glu to NH_2-R 7:3, α-chymotrypsin gave higher contents of MPy end groups. As observed above with thiophene and furan ring structures and here with pyridine, all of which are not

Table 2. Percent Yield and Oligo(γ-L-Et-Glu) Modified at the C-Terminus with R-NH_2 End-Capping Nucleophiles as a Function of the γ-L-Et_2-Glu to R-NH_2 Ratio, Enzyme Origin, and R-NH_2 Structure[a,b]

NH_2-R nucleophile	enzyme	γ-L-Et_2-Glu to NH_2-R nucleophile[c]					
		7:3[c]	5:5	3:7	7:3	5:5	3:7
		% yield[d]			% modified chains[d]		
BzA	papain	45	30	25	20	46	55
	bromelain	40	38	20	36	55	62
	α-chymotrypsin	30	8	0	30	0	0
	Multifect P-3000	0	0	0	0	0	0
	Purafect prime 4000 L	0	0	0	0	0	0
MPy	papain	50	35	20	17	30	40
	bromelain	55	35	30	15	18	26
	α-chymotrypsin	30	7	0	40	0	0
	Multifect P-3000	5	0	0	0	0	0
	Purafect prime 4000 L	3	0	0	0	0	0
NorbA	papain	70	51	np[e]	0	15	np[e]
	bromelain	60	40	np[e]	0	15	np[e]
	α-chymotrypsin	45	30	np[e]	0	18	np[e]
	Multifect P-3000	0	0	0	0	0	0
	Purafect prime 4000 L	0	0	0	0	0	0
F-OH	papain	50	42	15	20	30	45
	bromelain	57	50	10	0	20	30
	α-chymotrypsin	40	20	0	30	40	0
	Multifect P-3000	15	0	0	45	0	0
	Purafect prime 4000 L	8	0	0	0	0	0

[a] Reactions were carried out in 0.9 M phosphate buffer, for 3 h, at 40 °C and at the pH optimum for each enzyme used. [b] Yield % error was less than ±6%. [c] Underlined number gives the value in the ratio of NH_2-R. [d] Percent yield and modified chains was determined assuming oligopeptides consist of eight γ-Et-Glu repeat units. For products containing C-terminal modified moieties [oligo(γ-L-Et-Glu)-NH-R], the % yield was calculated by subtracting the weight contributed by C-terminal moieties as determined by ^{1}H NMR. Thus, % yield is total product yield [oligo(γ-L-Et-Glu) + oligo(γ-L-Et-Glu)-NH-R], where the contribution of NH_2-R to product weight is subtracted. [e] np = reaction not performed.

Scheme 3. Reaction Route with In Situ End-Capping Reagents Used for C-Terminal Functionalization of Oligo(γ-L-Et-Glu)



found in naturally occurring amino acids, the promiscuity of proteases to incorporate these non-natural structures as C-terminal groups is useful in diversifying the end-group structure and, therefore, potential functions of synthesized oligopeptides.

To further investigate the potential that it is the α-carboxyl group of *F* and *H* that results in their inability to function as amine nucleophiles for oligo(γ-L-Et-Glu) end-capping, L-phenylalaninol (*F*-OH) (Scheme 3.) was studied. *F*-OH is the reduced form of L-phenylalanine, so that -CH_2-OH replaces the anionic α-carboxyl group, whereas L-phenylalanine addition to L-Et_2-Glu did not produce end-capped oligo(γ-L-Et-Glu). For all five proteases studied herein, addition of *F*-OH to γ-L-Et_2-Glu in the monomer feed gave substantial quantities of oligo(γ-L-Et-Glu)-*F*-OH chains. Also, total precipitated product for γ-L-Et_2-Glu to L-phenylalaninol 7:3 remained high relative to total product yields reported for other NH_2-R compounds in Tables 1 and 2. Multifect P-3000 was particularly active in forming end-capped chains with Et_2-Glu to L-phenylalaninol (*F*-OH) 7:3. However, further increase in the γ-L-Et_2-Glu to L-phenylalaninol ratio to 5:5 for Multifect P-3000, and to 3:7 for α-chymotrypsin, resulted in no precipitated product. Hence, in these cases, *F*-OH was either a strong inhibitor of peptide synthesis or formed low molecular weight end-capped chains that did not precipitate. Indeed, earlier literature describes that peptide sequences bearing terminal heteroaromatic moieties can act as potent protease inhibitors.[40,41]

A series of experiments was performed using bicyclo[2.2.1]-5-heptene-2-methylamine (NorbA) as the amine nucleophile during oligo(γ-L-Et-Glu) synthesis. NorbA was synthesized by reduction of bicyclo[2.2.1]-5-heptene-2-carbonitrile following a literature procedure.[42] Using papain, bromelain, and α-chymotrypsin as protease catalysts and a γ-L-Et_2-Glu-to-NorbA ratio of 5:5 gave total product yields of 51, 40, and 30%, respectively. Analysis by ^{1}H NMR showed that the mol-% of

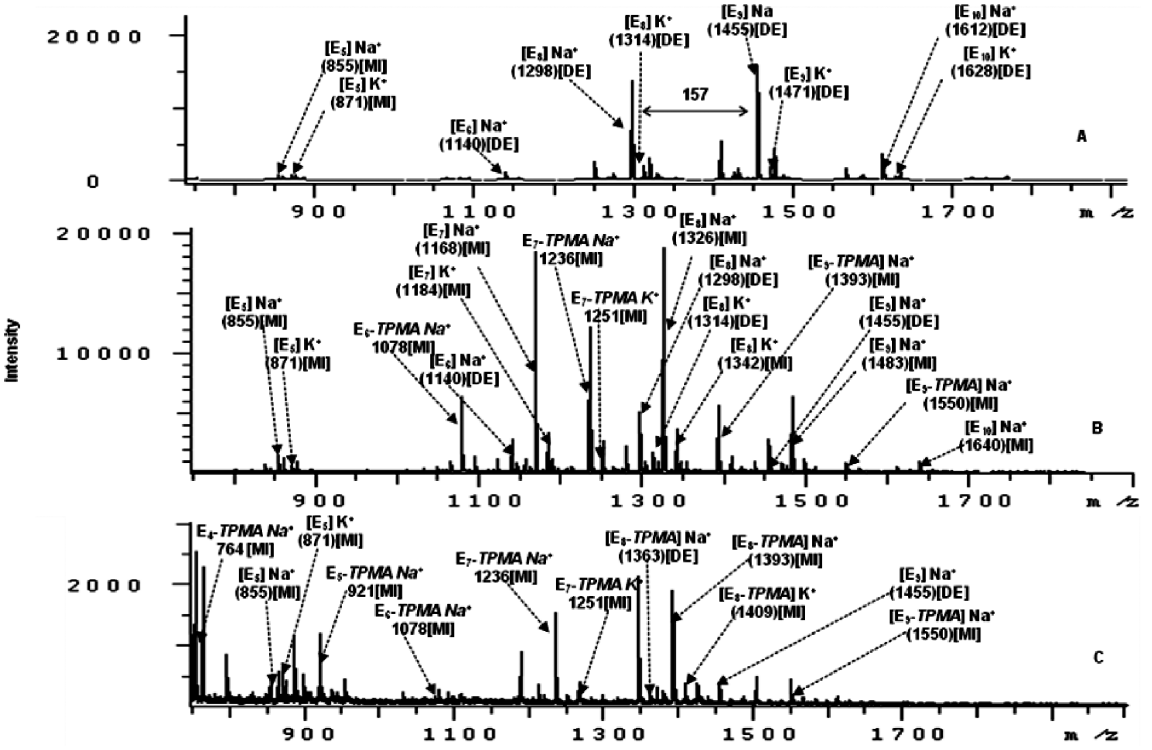


Figure 3. MALDI-TOF spectra of products consisting of oligo(γ-L-Et-Glu)[E_n] and oligo(γ-L-Et-Glu)[E_n]-NH-TPMA, where n represents the number of repeat units, prepared using the following proteases and γ-L-Et_2-Glu/TPMA molar feed ratios: (A) Multifect P-3000, no TPMA; (B) bromelain, 7:3; (C) Multifect P-3000, 7:3, [MI] molecular ion peak, [DE] desterified peak, the m/z values are $\pm$1 da of the expected m/z values.

oligo(γ-L-Et-Glu)-NH-NorbA chains were 15, 15, and 18%, respectively. In contrast to papain, bromelain, and α-chymotrypsin, Multifect P-3000 and Purafect prime 4000 L yielded no product even when the ratio of γ-L-Et_2-Glu-to-NorbA in the monomer feed was 7:3. The relatively low level of oligo(γ-L-Et-Glu)-NH-NorbA formed during oligopeptide synthesis compared to when other NH_2-R nucleophiles such as TPMA, FMA, MFMA, MPy, BzA, and *F*-OH were used is attributed to the bulky structure of the bycyclic NorbA ring structure.

MALDI-TOF Analysis of End-Capped Products. Oligo(γ-L-Et-Glu) catalyzed by Multifect P-3000 without addition of an R-NH_2 nucleophile produces a series of signals in MALDI-TOF spectrum Figure 3A corresponding to oligo(γ-L-Et-Glu)[E_n] where n represents the number of repeat units. For example, signals are observed at m/z 855 and 871, corresponding to the E_5 molecular ion peak associated with a sodium and potassium, respectively. In addition, signals corresponding to E_6 (1140), E_8 (1298, 1314), E_9 (1455, 1471), and E_{10} (1612, 1628) corresponds to (γ-L-Et-Glu) with one de-esterified γ-L-Glu unit, associated with a sodium and potassium ions, respectively. The highest intensity signals in MALDI-TOF spectrum in Figure 3A are observed for E_8 and E_9. This is in excellent agreement with DP_{avg} 9.5 determined by ^{1}H NMR for Multifect P-3000 catalyzed oligo(γ-L-Et-Glu) synthesis. The spectrum in Figure 3B was recorded of products synthesized by bromelain catalysis using a 7:3 molar ratio of L-Et_2-Glu-to-TPMA. Based on ^{1}H NMR, the product has a DP_{avg} of 8.3 and consists of a 50:50 mixture of oligo(γ-L-Et-Glu)-NH-TPMA and oligo-(γ-L-Et-Glu). As anticipated, the MALDI-TOF spectrum in Figure 3B shows identical signals that were listed and assigned above corresponding to oligo(γ-L-Et-Glu). The next set of peaks corresponding to oligo(γ-L-Et-Glu)-NH-TPMA molecular ion plus sodium are as follows: E_6 (1078), E_7 (1236), E_8 (1393), and E_9 (1550). Based on qualitative interpretation of the MALDI-TOF where the intensities of peaks at 1100 to 1350, 1400 to 1550, and 1600 to 1750 were compared, the percent oligo(γ-L-Et-Glu)-NH-TPMA to oligo(γ-L-Et-Glu) is 40 $\pm$ 10%. Thus, the extent of end-group capping with TPMA catalyzed by bromelain using a 7:3 molar ratio of γ-L-Et_2-Glu-to-TPMA, determined by ^{1}H NMR, is in excellent agreement with the MALDI-TOF results. The spectrum in Figure 3C was recorded of products synthesized by Multifect P-3000 catalysis using a 7:3 molar ratio of L-Et_2-Glu-to-TPMA. Based on ^{1}H NMR, the product has a DP_{avg} of 9.3 and consists of $>$95% of oligo(γ-L-Et-Glu)-NH-TPMA. Consistent with the ^{1}H NMR analysis, MALDI-TOF spectrum in Figure 3C shows only low intensity signals corresponding to oligo(γ-Et-Glu) E_5 (855, 871) and E_9 (1455) with one ester group de-esterified and associated with sodium and potassium ions, respectively. MALDI-TOF signals in Figure 3C corresponding to the molecular ion of oligo(γ-L-Et-Glu)-NH-TPMA associated with sodium or potassium ion are at E_6 (1078, 1094), E_7 (1236, 1251), E_8 (1393, 1409), and E_9 (1550). Based on qualitative interpretation of the MALDI-TOF spectrum in Figure 3C, where the intensities of peaks at 800 to 1000, 1100 to 1300, and 1400 to 1700 were compared, the percent of end-functionalized oligo(γ-L-Et-Glu)-NH-TPMA chains in the total product is 90 $\pm$ 5%. Hence, ^{1}H NMR and MALDI-TOF values of end-group capping with TPMA, catalyzed by Multifect P-3000, using a 7:3 molar ratio of γ-L-Et_2-Glu-to-TPMA, are in excellent agreement. The absence of low intensity peaks corresponding to one de-esterified ester group for oligo(γ-L-Et-Glu)-NH-TPMA in Figure 3C suggests that de-esterification occurs at the C-terminal oligo(γ-L-Et-Glu) unit, not at other sites along the oligomer. In other words, if protease-catalyzed de-esterification occurred at sites other than the C- terminal unit, then nearly quantitative end-capping at this position could occur along with de-esterication giving the corresponding molecular ions in the MALDI-TOF spectrum.

Summary of Results

This paper describes an innovative one-pot biotransformation for preparation of oligo(γ-L-Et-Glu) decorated at the C-terminus with amine-functionalized end-groups. Papain, bromelain, α-chymotrypsin, Multifect P-3000, and Purafect prime 4000 L were used as catalysts for oligomerization of γ-L-Et_2-Glu in the presence of monofunctional amines. Protease concentrations in reactions were normalized based on their activity for casein hydrolysis. The relative order of protease activities for oligo(γ-L-Et-Glu) synthesis is as follows: papain ~ bromelain > α-chymotrypsin > Multifect P-3000 > Purafect prime 4000 L at pH optima values of for the proteases at 8, 8, 9, 9, and 9, respectively. Irrespective of the protease studied, addition of increased quantities of amine nucleophile relative to Et_2-Glu results in decreased % yield but increased mol % of amine end-capped chains oligo (γ-L-Et-Glu)-NH-R. These trends were explained by that, increasing the ratio of NH_2-R: γ-L-Et_2-Glu groups: (*i*) can cause an increase in the quantity of chains formed and, correspondingly, a reduced oligo(γ-L-Et-Glu)-NH-R DP_{avg}, resulting in product that precipitates to a lesser extent and (*ii*) results in a reduced concentration of oligomerizable substrate in reactions. It also may be that NH_2-R functions as a competitive inhibitor of protease catalysts.

Regardless of the protease used, TPMA was the most efficient NH_2-R compound for reacting with α-ethyl ester moieties of γ-L-Et_2-Glu monomer or propagating oligo(γ-L-Et-Glu) chains thereby forming C-terminal functionalized oligopeptides. For all NH_2-R compounds investigated herein data was obtained on how their efficiency for end-group functionalization differed as a function of the protease used. For example, with Et_2-Glu to TPMA of 7:3, the mol % of oligo(γ-L-Et-Glu)-TPMA formed with papain, bromelain, α-chymotrypsin, Multifect P-3000, and Purafect prime 4000 L is 25, 50, 75, >95, and >95, respectively.

Work then addressed the extent of protease promiscuity as a function of NH_2-R structure. This provided insights into the range of amine nucleophiles that may be incorporated at oligo(γ-L-Et-Glu) C-termini. For example, TPMA and TPEA differ structurally by just one methylene unit between thiophene and amine groups. But TPEA was a relatively poor amine nucleophile for oligo(γ-L-Et-Glu) end-capping. Indeed, only papain, with γ-L-Et_2-Glu to TPEA of 7:3 and 5:5, gave 20 and 25 mol % of oligo(γ-L-Et-Glu)-TPEA chains. Hence, for this series of amine nucleophiles and proteases, there appears to be a strong preference for using α- instead of β-amino acids. Using FMA in place of TPMA as the amine nucleophile probed the subtle change of replacing a thiophene with a furan heterocylic ring structure. While total product yields were similar with both these nucleophiles, TPMA was more efficient than FMA for C-terminal end-capping reactions. The affect of incorporating a methyl substituent at FMA furan ring 5-positions (e.g., using MFMA) was also investigated. For all five proteases, total yields were lower when using MFMA instead of FMA as the amine nucleophile. However, in general, the efficiency of MFMA and FMA for C-terminal modification based on precipitated product was similar. In fact, for papain and γ-L-Et_2-Glu to R-NH_2 5:5, incorporation of MFMA was higher than FMA (44 vs 20%).

Intriguingly, attempts to use either L-histidine or L-phenylalanine as amine nucleophiles were unsuccessful. To explore what structural characteristic of these compounds might result in this outcome, two L-phenylalanine analogs were tested, where the α-carboxyl group was replaced by a hydrogen atom (BzA) and a CH_2-OH moiety (*F*-OH), respectively. At molar ratio of γ-L-Et_2-Glu-to-BzA of 3-to-7, the mol % of oligo(γ-L-Et-Glu)-BzA with papain and bromelain was 55 and 62%, respectively. At 7:3 L-Et_2-Glu to *F*-OH, catalysis by papain, α-chymotrypsin and Multifect P-3000 formed oligopeptides where oligo(γ-L-Et-Glu)-*F*-OH constituted 20, 30, and 45 mol % of total product. Thus, based on this limited set of subtrates and enzymes, it appears that the presence of an α-carboxyl group on the amine nucleophile was deleterious to its function for amide formation with either γ-L-Et_2-Glu or propagating oligo(γ-L-Et-Glu) chains.

Typically, we have found that, in the absence of an amine nucleophile, protease-catalyzed synthesis of oligo(γ-L-Et-Glu) occurred where MALDI-TOF analysis showed the formation of a substantial fraction of chains hydrolyzed at one of their ethyl ester pendant or end-group sites. If the site of ester hydrolysis is the C-terminus, then complete modification of the C- terminus by an amine nucleophile would give products with all other esters intact. Indeed, for oligo(γ-L-Et-Glu)-NH-TPMA synthesized by Multifect P-3000 catalysis, MALDI-TOF signals lacked low intensity peaks corresponding to one de-esterified ester group for oligo(γ-L-Et-Glu)-NH-TPMA. Therefore, we believe that de-esterification observed primarily occurs at the α-carboxyl moiety at the C- terminus of oligo(γ-L-Et-Glu).

The cumulative results reported herein provide a unique general method that is simple and scalable by which oligopeptides can be prepared from protease catalysis from one or more amino acid alkyl esters in one-pot reactions with control of end-group structure. Furthermore, insights gained into protease promiscuity will allow the design of amine nucleophiles with desired functionality and high reactivity for incorporation at oligo(γ-L-Et-Glu) C-termini.

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Supporting Information Available: Hydrolytic activity and protein content of proteases used; MALDI-TOF spectra of products formed using the following amine nucleophiles: 1-phenyl methanamine (BzA), 2-amino methylfuran (FMA), 5-methyl-2-amino methylfuran (MFMA), 4-methylamino pyridine (MPy), bicyclo[2.2.1]hept-5-en-2ylmethanamine (NorbA), and L-phenylalaninol (FOH). This material is available free of charge via the Internet at http://pubs.acs.org.

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