Protease-Catalyzed Co-Oligomerizations of L-Leucine Ethyl Ester with L-Glutamic Acid Diethyl Ester: Sequence and Chain Length Distributions

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ABSTRACT: Four proteases (papain, bromelain, α-chymotrypsin and protease SG) were used to catalyze co-oligomerizations of L-leucine ethyl ester (L-Et-Leu) with diethyl-L-glutamate (L-$(Et)_2$-Glu). Protease added to reaction was normalized based on their relative activities for casein hydrolysis. With the exception of papain that shows a very broad pH optimum for oligo(γ-Et-L-Glu) synthesis, the pH optimum for peptide synthesis was above that for peptide hydrolysis. For L-$(Et)_2$-Glu/L-Et-Leu (1:1 mol/mol) co-oligomerizations, the relative order of activities of the four proteases is papain ≈ bromelain > α-chymotrypsin > protease SG. ^{1}H NMR analyses showed that co-oligopeptide compositions were close in value to the monomer feed ratio. Reactivity ratios, r_{Leu} and r_{Glu}, are nearly identical and have values close to one, suggesting propagation during oligomer synthesis tends to occur randomly giving random sequences along oligopeptides. MALDI-TOF spectra information was obtained on both chain length distribution and chemical composition distribution for oligo(γ-Et-L-Glu-*co*-∼50mol%-L-Leu) prepared using papain, bromelain, α-chymotrypsin or protease SG as catalysts. From MALDI-TOF generated total abundance intensities, DP_{avg} values of these samples are 7.5, 6.1, 8.5 and 8.7 respectively, which agrees well with values determined by ^{1}H NMR (7.3, 6.5, 8.2 and 8.5 respectively). Oligo(γ-Et-L-Glu-*co*-∼50mol%-L-Leu), synthesized using α-chymotrypsin and protease SG, consists of the broadest chain length distributions (5−12 and 6−11, respectively). In contrast, oligopeptides from bromelain and papain catalysis have narrower chain length distributions (5−8 and 6−9, respectively). Also, MALDI-TOF shows a large heterogeneity of leucine contents for oligo(γ-Et-L-Glu-*co*-∼50mol%-L-Leu) synthesized by all four protease catalysts, consistent with co-oligomerizations occurring by random propagation events. Eight tripeptides representing all possible sequences were prepared by standard solid-state Fmoc-based peptide chemistry. Oligo(γ-Et-L-Glu-*co*-∼50mol%-L-Leu) chains prepared using all four proteases were degraded to oligomers where the primary constituent in the population was trimers. Using LC−MS to analyze the relative content of tripeptide sequences, and calculating the abundance of triad sequences for a statistically random process, it was found for all four proteases that experimental and theoretical values are in excellent agreement. Hence, in agreement with MALDI-TOF and reactivity ratio results, LC−MS analysis showed that random co-oligopeptides were formed. In other words, for this set of monomers and proteases, no preference or selectivity was observed for addition of L-$(Et)_2$-Glu or L-Et-Leu to propagating oligomer chains.

Introduction

Specially designed oligopeptides on their own, or conjugated with lipids or PEG, are of intense interest due to their ability to: (i) self-assemble into interesting nanostructures,[1,2] (ii) biological activity and associated uses in cosmetics[3] and regenerative medicine,[4] and (iii) specific binding to various substances such as metals.[5] Molecular self-assembly for the design and fabrication of nanostructures is leading to exciting developments in advanced materials, scaffolds and a wide range of applications in medical technologies such as regenerative medicine and drug delivery systems.[6] Zhang and co-workers reported self-complementary peptides that spontaneously self-assemble to form interwoven nanofibers in the presence of monovalent cations.[7,8] These nanofibers further form a hydrogel consisting of greater than 99.5% water. The constituent of the hydrogel scaffold is made of peptides with alternating hydrophilic and hydrophobic amino acids. Self-assembling peptide scaffolds have shown promise as substrates for tissue-cell attachment and growth in a three-dimensional environment.[9] Aggeli et al.[10] designed short peptides that self-assemble in nonaqueous media forming long, semiflexible, polymeric beta-sheet peptide nanotapes. Sylvain et al.[8] designed another class of amphiphilic surfactant-like peptides. These 7−8-residue peptides, each 2 nm in length, have properties very similar to those observed in biological surfactant molecules. They have a hydrophilic aspartic acid headgroup at the C terminus, and a lipophilic tail made of hydrophobic amino acids such as leucine and valine. It is anticipated that further work into peptides that self-assemble and have surfactant properties will lead to important new materials. However, current synthetic routes to oligopeptides are tedious as discussed below.

The most widely used approaches to prepare peptides are solid phase peptide synthesis (SPPS) and liquid phase peptide synthesis (LPPS). These are well established methods that provide high purity peptides with precise control of sequence and chain length. However, these chemical methods are costly since they involve toxic reagents, multiple steps with protection−deprotection chemistry, and product purification.[11] An alternative approach is preparation of oligopeptides by recombinant DNA technology. This involves the design of genes that encode protein−oligopeptide conjugates that can be produced via fermentation.[12] Subsequently, oligopeptides are obtained by their specific cleavage of protein−oligopeptide conjugates and subsequent purification. By this approach oligopeptides can be prepared with complete control of sequence and chain length without use of toxic reagents. Current drawbacks of this fermentative route to oligopeptides are low expression efficiencies of protein−oligopeptide

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conjugates and, therefore, low product yields. Consequently, oligopeptides prepared via these chemical and fermentative methods are costly, limiting their use and concentrations in products.

An alternative route to oligopeptides is by protease-catalysis of amino acid esters. Positive attributes of protease-catalyzed oligopeptide synthesis include (i) avoid racemization, (ii) activation requires simple formation of ethyl esters, (iii) regioselectivity without specific protection of α- vs γ-carboxylic acids, and (iv) mild nonhazardous operating conditions.[12,13] Protease catalyzed oligopeptide synthesis is equilibrium or thermodynamic controlled in order to reverse proteolysis. Reaction conditions that lead to product precipitation or extraction increase efficiency of the reverse reaction.[13] For serine and cysteine proteases that form activated acyl enzyme intermediates during catalysis, kinetically controlled syntheses have proved useful. This involves the use of simple amino acid esters that function as activated acyl donors thereby accelerating reaction rates.[12] Studies described herein make use of principles from both kinetic and thermodynamically controlled reactions in that reactants are activated by formation of esters and products precipitate from reactions. Since protease-catalyzed routes to oligopeptides can be carried out in aqueous media from simple building blocks, they are an attractive alternative to solid and liquid phase peptide synthetic methods.

The following summarizes important studies that have shown the feasibility of using proteases to prepare oligopeptides. Aso et al.[14] reported protease-catalyzed oligomerization of dialkyl L-glutamate hydrochloride. Uemura et al.[15] increased the organosolubility of papain by its modification with poly(ethylene glycol), PEG. They then used PEG-conjugated papain to catalyze oligomerizations of dialkyl L-aspartate and dialkyl L-glutamate in benzene and thereby prepared oligomer mixtures with chain lengths from heptamer to decamer. Ester hydrochlorides of methionine, phenylalanine, threonine, and tyrosine were polymerized by papain catalysis in buffer, giving poly([α]-amino acid)s with average degree of polymerization (DP_{avg}) less than 10.[16−18] Uyama et al.[19] used the proteases papain, bromelain, and α-chymotrypsin as catalysts for homo- and co-oligomerizations of diethyl L-glutamate hydrochloride. Analysis of $^1H-^1H$ COSY NMR recorded of these oligopeptides led Uyama et al.[19] to conclude that oligomers formed consist exclusively of α-linked γ-ethyl glutamate units. Matsumura et al.[20] investigated oligomerization of diethyl L-aspartate in bulk using the alkalophilic protease from *Streptomyces* sp. as catalyst. Analysis of the product formed showed it consisted of 88% α-linkages. Oligomerization of diethyl L-aspartate was also reported by Soeda et al.[21] using microbial protease BS as catalyst in solutions containing small volumes of water. For example, by conducting protease BS catalyzed diethyl L-aspartate oligomerizations at 40 °C, for 2 days, in MeCN containing 4.5 vol % water, α-linked poly(β-ethyl L-aspartate) was prepared in 85% yield and M_w up to 3700.

Often neglected in previous studies of protease-catalyzed oligopeptide synthesis is knowledge acquired during plastein-type reactions. Plastein reactions involve peptide bond forming reactions or resynthesis of proteins from protein hydrolysates.[22,23] For example, it was discovered that the pH optima for protease-catalyzed peptide bond formation differs from that of protease-catalyzed peptide bond hydrolysis. However, many previous studies concerned with protease-catalyzed oligopeptide synthesis neglected to determine the optimum pH for synthetic reactions and, instead, used pH optima defined for hydrolytic peptide reactions.[14,19,20] Changes in reaction pH were not considered even though decreases in pH during oligomerizations may reduce or deactivate protease catalysts.[24] Furthermore, comparisons of proteases were not based on common, well-defined units of activity.[15,19,20] Without such information it becomes impossible to repeat these studies. Moreover, for co-oligomerizations, information on relative reactivity of comonomers was not given, only the average composition of co-oligopeptides.[19] Indeed, co-oligopeptide sequence distribution will ultimately control properties of these materials. Consideration of the above prompted an earlier study by our laboratory that reinvestigated papain-catalyzed oligomerizations of diethyl L-glutamate hydrochloride.[24] Papain activity was defined using Ac-Phe-Gly-*p*-nitroanaline hydrolysis. By conducting oligomerizations in 0.9 M phosphate buffer at 40 °C, oligo(γ-ethyl-L-glutamate) was rapid giving product in 80% yield by 10 min. As long as the pH in reaction media was maintained between 5.5 and 8.5, papain activity remained high giving product in >60% yield. Furthermore, there was no significant effect of ionic strength on oligopeptide yield and the dominant role of phosphate buffer was its ability to control reaction pH.

This paper describes protease-catalyzed co-oligomerizations of γ-ethyl-L-glutamate with L-leucine ethyl ester. This system was selected due to its potential to prepare amphililic co-oligopeptides that would lead to biosurfactants and other self-assembling systems. The activity of protease catalysts (papain, bromelain, α-chymotrypsin and protease SG) for co-oligomerizations was first characterized by the casein hydrolysis assay. Oligopeptide synthesis activity as a function of pH was determined for diethyl-L-glutamate homopolymerizations, and optimal pH values were then used for co-oligomerizations. Since co-oligomers can be complex mixtures with respect to oligomer length, amino acid composition and repeat unit sequence, traditional methods of peptide sequence analysis are ineffective for their characterization. In this study monomer relative reactivity ratios were determined to evaluate the apparent specificity of proteases for addition of either L-Et-Leu or L-$(Et)_2$-Glu to propagating chain ends. Relative reactivity ratios provided initial evidence of whether copolymer sequence tends toward block, alternating or random distributions. Further information on oligomer sequence distribution was provided by MALDI-TOF spectroscopy. Moreover, all possible combinations of Leu-Glu tripeptide sequences were prepared by solid-state chemical methods. These pure tripeptide standards were used to determine sequence distribution of hydrolyzed co-oligopeptides at the triad level by LC−MS. MALDI-TOF also provided valuable insight into the chain-length distribution of co-oligopeptides. The cumulative results of this paper provide important insights into chain length and sequence specificity of papain, bromelain, α-chymotrypsin and protease SG for L-Et-Leu/L-$(Et)_2$-Glu co-oligomerizations.

Experimental Section

Materials. L-Glutamic acid diethyl ester hydrochloride (L-$(Et)_2$-Glu·HCl), L-leucine ethyl ester hydrochloride (L-Et-Leu·HCl) were purchased from Tokyo Kasei Co. Ltd. in the highest available purity and used as received. Crude papain (cysteine protease; EC 3.4.22.2; source, *Carica papaya*; 30 000 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; 2.290 units/mg solid; 3.650 units/mg protein), α-chymotrypsin (serine protease; EC 232-671-2; source, bovine pancreas, type II, 83.9 units/mg solid; 96 units/mg protein), Protease SG (serine protease; EC 232-909-5; source, S*treptomyces griseus* type XIV, 5.8 units/mg solid) were purchased from Sigma Aldrich Inc. and used as received. *N*-Hydroxybenzotriazole (HOBT), *O*-benzotria-

zole-*N*,*N*,*N*,*N*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) were purchased from VWR International Inc. and were used as received. Also, *N*,*N*-diisopropylethylamine (DiEA), dimethylformamide (DMF), trifluoroacetic acid (TFA), triisopropylsilane (TIS), and anisole were purchased from Sigma-Aldrich Co. and were used as received. Folin & Ciocalteu's phenol reagent (F−C) was prepared by diluting 10 mL of Folin & Ciocalteu's phenol reagent purchased from Sigma Aldrich Inc. to 40 mL with deionized water. Enzyme diluent was prepared from 10 mM sodium acetate buffer and 5 mM calcium acetate with adjustment of the pH to 7.5 at 37 °C with 0.1 M acetic acid or 0.1 M NaOH.

Methods. *General Procedure for Protease-Catalyzed Oligo(γ-Et-L-Glu) Synthesis.* The method for oligo(γ-Et-L-Glu) synthesis was performed as previously described in the literature.[24] In summary, L-glutamic acid diethyl ester hydrochloride (600 mg, 2.5 mmol), protease (16 units/mL), and 5 mL of phosphate buffer solution set at a predetermined pH were transferred to a 15 mL Erlenmeyer flask. The flask was gently stirred in a water bath at 40 °C for a predetermined reaction time. Then, the reaction mixture was cooled to room temperature and deionized water (20 mL) was added. The insoluble product was separated by centrifugation (6000 rpm) and washed once with dilute HCl (2% v/v) solution and then twice with deionized water. The resulting product was lyophilized, giving a beige powder (powder color varied as a function of the protease used).

General Procedure for Protease-Catalyzed Co-Oligomerization of L-(Et)$_2$-Glu·HCl and L-Et-Leu·HCl. A mixture of L-(Et)$_2$-Glu·HCl(1.25 mmol), L-Et-Leu·HCl (1.25 mmol), protease (16 units/mL) and 5 mL of phosphate buffer solution (pH 8.0, concentration 0.9 M) was transferred to a 50 mL round-bottom flask. The mixture was gently stirred in a water bath at 40 °C for 4 h. Then, the reaction mixture was cooled to room temperature by addition of 20 mL of deionized water. The precipitate was collected by centrifugation (6000 rpm) and washed once with dilute HCl (2% v/v) solution and then twice with deionized water. The resulting material (product) was lyophilized, giving a beige powder (powder color varied with protease used).

Peptide De-Esterification and Degradation. Dried oligopeptide powder (100 mg) was kept in 1 N NaOH solution (5 mL) at 60 °C for 36 h; the mixture was neutralized with 6.0 M hydrochloride solution. Subsequently, the peptide was passed through a Sephadex G-10 desalting column using an aqueous 1 wt % acetic acid elution phase and ninhydrin solution as color indicator. The collected eluent with oligopeptides was lyophilized to give a white powder. This powder (10 mg) was then transferred into a Pierce vacuum hydrolysis tube which contains boiling hydrochloric acid (2.5 mL, 6 N), the solution was maintained at 110 °C (Pierce Reacti-Therm module) for 60 min, and then the degraded hydrolysate was lyophilized to give a white powder for LC-MS analysis.

Preparation of Tripeptide Standard Compounds for LC−MS Analysis. All possible combinations of Leu/Glu tripeptide sequences (total of eight) were chemically synthesized following well-established solid-state protocols.[11] In summary, fluorenylmethoxycarbonyl (Fmoc) peptide chemistry was performed using an ABI 430A automatic peptide synthesizer (Applied Biosystems, Foster City, CA). Acylation was performed by reaction of the N-termini of peptides with 2 equiv of HOBT, 2 equiv of HBTU, and 4 equiv of DiEA in DMF at room temperature. Peptide products were cleaved from the resin by exposure to a TFA−TIS−anisole−water (91:3:3:3 by volume) solution at 0 °C for 2 h. The peptide-containing solution was collected via vacuum filtration and dried in a rotary evaporator (37 °C, <25 mmHg). The product was precipitated by addition of cold diethyl ether, centrifuged at 3000 rpm for 5 min, filtered, dried under vacuum, and stored at 0 °C. Crude peptides were dissolved in deionized water at 2% (w/w) and purified by reversed-phase high performance liquid chromatography (HPLC) with a Varian peptide C18 (20 mm × 250 mm, GL Sciences Inc.) semipreparative column using aqueous 0.1% TFA and an acetonitrile gradient as the mobile phase. After lyophilization, the peptides were obtained as white powders or crystals, depending on the tripeptide composition.

Determination of Protease Hydrolytic Activity. Determination of protease activity followed a method described in the literature.[25] In summary, to a 2 mL Eppendorf tube were transferred 25 μL of protease solution (0.1 unit/mL of protease dissolved in the enzyme diluent described above) and 130 μL of 0.65% (w/v) casein solution in 50 mM potassium phosphate buffer at pH 7.5. The resulting solution was incubated at 37 °C for 10 min. Subsequently, 130 μL of a 110 mM solution of trichloroacetic acid solution was added and this solution was further incubated for 20 min at 37 °C. Then, the solution was centrifuged and the supernatant (250 μL) was mixed with 625 μL of 500 mM $NaCO_3$ solution and 125 μL of Folin & Ciocalteu's phenol reagent (see Materials section) at 37 °C. The absorption was measured at 660 nm using a Shimadzu UV-1601. An activity unit is defined herein as a quantity of enzyme that liberates 1 μmol of tyrosine equivalents per min per mL in pH 7.5 buffer solution at 37 °C.

Instrumental Methods. *Nuclear Magnetic Resonance (NMR) Spectroscopy.* Proton (^{1}H) NMR spectra were recorded on a Bruker DPX 400 spectrometer at 400 MHz. NMR experiments were performed in DMSO-d_6 at 10 mg/mL with a data acquisition delay of 1 s and a total of 128 scans. Data was collected by software BioSpin and analyzed by data processing software: MestRe-C. Proton chemical shifts were referenced to tetramethylsilane (TMS) at 0.00 ppm.

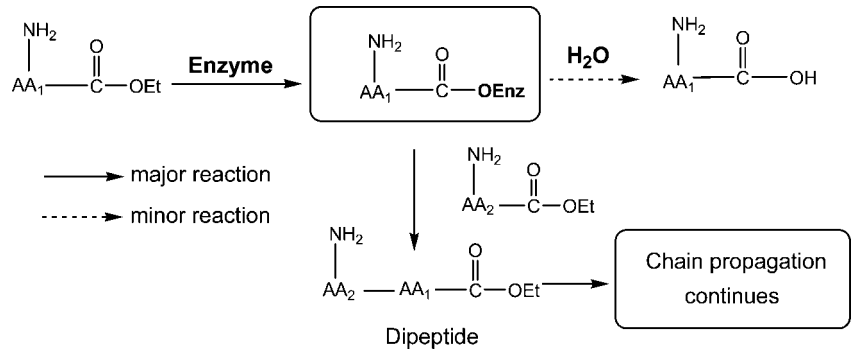
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF). MALDI-TOF spectra were obtained on an OmniFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Inc.). The instrument was operated in a positive ion linear mode with an accelerating potential of +20 kV. The TOF mass analyzer had pulsed ion extraction. The linear flight path was 120 cm. OmniFLEX TOF control software was used for hardware control and calibration. Spectra were acquired by averaging at least 200 laser shots. The pulsed ion extraction delay time was set at 200 ns. The spectrometer was externally calibrated using angiotensin II as a standard (1046.54 amu). To generate the matrix solution, a saturated solution of α-cyano-4-hydroxycinnamic acid (CCA) was prepared in trifluoroacetic acid/acetonitrile (TA, 1 to 10 v/v). Oligopeptide samples dissolved in dimethyl sulfoxide (DMSO, 5 μL) were diluted with TA solution to 1−5 pmol/μL and mixed with 5 μL of saturated matrix solution. Then, 1 μL of this mixture was applied onto the clean target. The sample target was dried in a stream of cold air from a dryer. The abundance intensities of peaks vs *m*/*z* were collected via X-massOminFLEX 6.0.0 software and then were exported to an MS Excel spreadsheet for further calculations. The relative intensity threshold was set so that peaks with intensity values less than 5% of the highest peak were considered as noise and removed from the database.

LC−MS Analysis. Lyophilized hydrolysate was dissolved in the mobile phase solution (1 mg/mL) for LC analysis. The solvent delivery system was a Waters Alliance 2795 Separation Module (Milford, MA) coupled with a Waters 2996 photodiode array detector and Waters ZQ detector with an electron spray ionization mode. The separation was carried out on a reversed-phase column (Polymer Laboratory PLRP-S) that is 250 × 2.1 mm with a particle size of 5 μm. The mobile phase used for separation contained 10% H_2O, 85% acetonitrile, and 5% TFA solution at flow rate 1 mL/min. Data were collected and analyzed by MassLynx software.

Results and Discussion

For protease catalyzed peptide synthesis, the kinetic model mechanism[26,27] (see Scheme 1) reveals that, prior to peptide bond formation, activated amino acid and enzyme interact forming a Michaelis−Menten complex (i.e. acyl enzyme intermediate). This complex is then competitively deacylated by water and a nucleophile. If the nucleophile is an amino acid or oligopeptide, a new peptide bond is formed. Precipitation of oligopeptides from the reaction solution during synthesis further shifts the equilibrium toward peptide formation.[13] To normalize

Scheme 1. Mechanism for Peptide Bond Formation[a]



[a] See ref 27.

Table 1. Co-Oligomerization of L-(Et)$_2$-Glu/L-Et-Leu Using 1:1 Molar Feed Ratios and Papain, Bromelain, α-Chymotrypsin and Protease SG as Catalysts

samples	proteases	protein content[a] (μg/mg)	sp act. × 10^3 (unit)[b]	feed ratio (mol) [Leu]$_o$/[Glu]$_o$	prod ratio (mol) [Leu]/[Glu][c] ± 3	yield (mol %)[d]	DP[c] ± 0.4
I	papain	405	1.98	50/50	52/48	68 ± 2	7.3
II	bromelain	573	1.50	50/50	55/45	69 ± 1	6.5
III	α-chymotrypsin	930	1.03	50/50	53/47	14 ± 1	8.2
IV	protease SG	690	3.91	50/50	54/46	7 ± 1	8.5

[a] Protein contents were determined by the BCA method. [b] Activity unit is the amount of enzyme activity that catalyzes the release of 1 μmol of tyrosine equivalents per min per mL in buffer solution at 37 °C. [c] Determined by ^{1}H NMR. [d] Values reported are the mean from at least duplicate experiments, and errors define the maximum and minimum values obtained.

the activity of the four different proteases studied herein, casein was used for colorimetric protease activity assay (see Experimental Section).[25,28] Furthermore, to obtain specific activities for these proteases, protein concentration was determined by the BCA method.[29] The results of protein content and activity measurements for these four proteases are listed in Table 1. 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 in buffer at 37 °C) of protease was used to catalyze homo-oligomerizations of L-(Et)$_2$-Glu and co-oligomerizations of L-(Et)$_2$-Glu and Et-L-Leu.

Optimum pH for Protease-Catalyzed Oligo(γ-Et-L-Glu) Synthesis. Previous studies on plastein reactions[30] demonstrated that the pH optima for protease-catalyzed peptide bond hydrolysis and synthesis can differ substantially. While literature values are available of pH optima for peptide hydrolysis reactions for all proteases studied herein, pH optima for bromelain, α-chymotrypsin and protease SG catalyzed oligopeptide synthesis reactions were previously unknown. Li et al.[24] investigated the pH−activity relationship for papain-catalyzed L-(Et)$_2$-Glu oligomerizations and reported that a broad plateau exists, between pH 5.5 and 8.5, where papain activity remained high (>80%).

Determination of pH optima values for bromelain, α-chymotrypsin and protease SG catalyzed oligopeptide synthesis were investigated herein. Using L-(Et)$_2$-Glu oligomerizations as the model reaction, reactions were performed using 16 units/mL of protease, in 0.9 M phosphate buffer, at 40 °C, for 4 h at initial pH values of 6, 7, 8, 9 and 10 (see Figure 1). For bromelain, the yield of precipitated oligo(γ-Et-L-Glu) increased from 27 to 81 ± 4% as the pH increased from 6 to 8. Further increase in pH to 9 resulted in no substantial change in yield, but the yield decreased to 72% at pH 10. In comparison to bromelain, pH changes for α-chymotripsin catalyzed oligomerization of L-(Et)$_2$-Glu had less dramatic effects on oligo(γ-Et-L-Glu) yield. The optimum yield using α-chymotrypsin was 49 ± 3% at pH 9. Of the four proteases, protease SG showed the lowest activity for L-(Et)$_2$-Glu oligomerization. The optimum yield using protease SG was 12 ± 2% at pH 9. In contrast, the specific activity of protease SG (Table 1) for casein hydrolysis was nearly more than two times higher than the other proteases studies.

Overall, based on oligo(γ-Et-L-Glu) yield during 4 h oligomerizations, the relative order of activities for the four proteases is as follows: papain[24] ≈ bromelain > α-chymotrypsin > protease SG. Furthermore, for this limited set of proteases, cysteine-type proteases (papain and bromelain) were more active than serine-type proteases (α-chymotrypsin and protease SG). The optimum pH values for peptide hydrolysis using papain, bromelain, α-chymotrypsin and protease SG are 6.0−7.0, 4.5−5.5, 7.0 and 8.0, respectively.[31,32] Hence, with the exception of papain that shows a very broad pH optimum for oligo(γ-Et-L-Glu) synthesis, the pH optimum for peptide synthesis was above that for peptide hydrolysis. A contributing factor that offers an explanation for this phenomenon is as follows: since a significant proportion of amino acid α-amino groups are in their uncharged form at alkaline pH values, this would facilitate nucleophilic attack to form peptide bonds.[33,34]

L-(Et)$_2$-Glu/L-Et-Leu Co-Oligomerizations and Reactivity Ratio Determinations. Table 1 lists co-oligomer yields, compositions and DP$_{avg}$ for L-Leu/γ-Et-L-Glu co-oligomers, prepared using papain, bromelain, α-chymotrypsin and protease SG from a 1:1 monomer feed ratio. Based on qualititative inspection of the MALDI-TOF spectrum for oligo(L-Leu-*co*-48% γ-Et-L-Glu) (Figure 3), prepared by papain catalysis (sample I), it appears that the compositional distribution of oligomers tends toward random. By using cysteine-type proteases papain and bromelain, oligomers were obtained in high yields (nearly 70%). In contrast, yields with α-chymotrypsin and protease SG were 14 ± 2 and 7 ± 2%, respectively. Thus, for L-(Et)$_2$-Glu/L-Et-Leu co-oligomerizations, the relative order of activities of the four proteases is as follows: papain ≈ bromelain > α-chymotrypsin > protease SG. This is precisely the same order of activities for these enzymes determined for L-(Et)$_2$-Glu homo-oligomerizations. Further inspection of Table 1 shows that amino acid compositions, from ^{1}H NMR analyses (see below) of co-oligopeptide samples I−IV, all have values close to the monomer feed ratio (1:1). However, DP$_{avg}$ values,

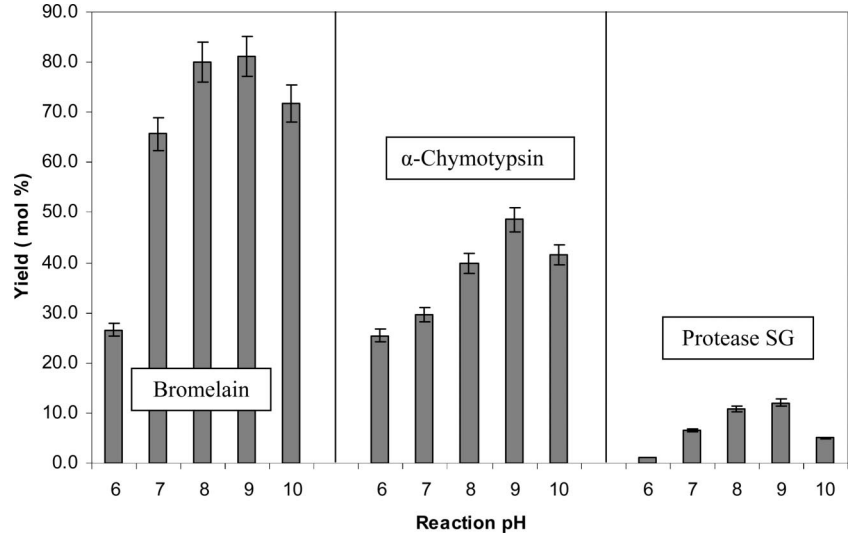


Figure 1. Synthetic activities of papain, bromelain, α-chymotrypsin and protease SG at different pH values using 0.5 M L-$(Et)_2$-Glu·HCl, 16 units/mL catalyst, 0.9 M phosphate buffer, at 40 °C, for 4 h. Values reported are the mean from at least duplicate experiments, and error bars define the maximum and minimum values obtained.

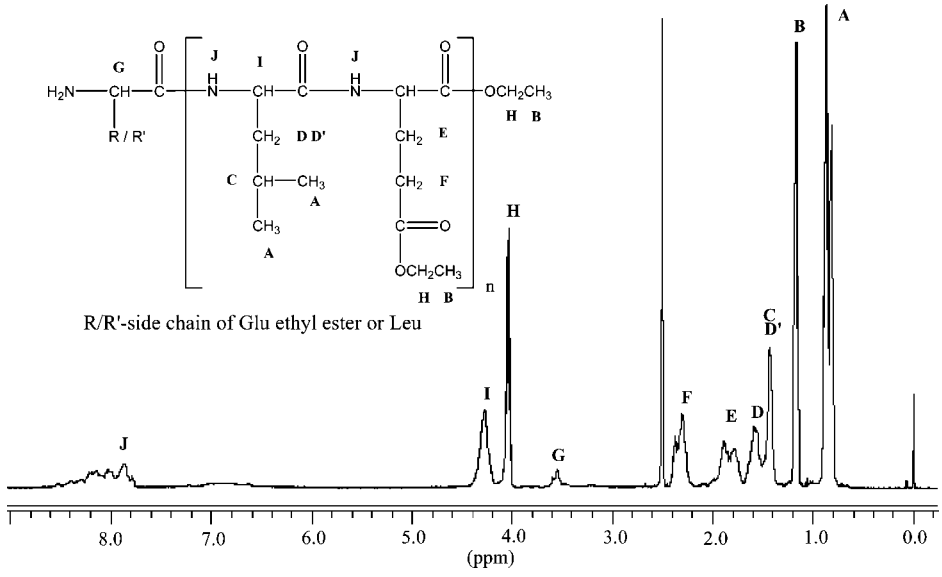


Figure 2. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of oligo(γ-Et-L-Glu-*co*-L-Leu) synthesized using 0.25 M L-$(Et)_2$-Glu·HCl and 0.25 M L-Et-Leu·HCl, 16 units/mL papain, at 40 °C for 4 h, 0.9 M phosphate buffer, pH 8.5.

calculated from ^{1}H NMR, were one or two units shorter for oligomers prepared by papain and bromelain (7.3, 6.5 respectively) relative to those prepared using α-chymotrypsin and protease SG (8.2, 8.5 respectively). These differences in average chain length could be due to differences in compositional and/or sequence distributions of products (see discussion below).

To further characterize the relative reactivities of papain, bromelain, α-chymotrypsin and protease SG for L-$(Et)_2$-Glu/L-Et-Leu co-oligomerizations, reactivity ratios were determined by the Mayo−Lewis slope−intersection method[35] (Table 2). This required that precipitate yields were kept below 10% to minimize effects of changes in monomer concentration ratio during reactions. This was accomplished by terminating reactions at 10 min by separation of the precipitate and aqueous phase via filtration and washing the precipitate with deionized water (see Experimental Section). Indeed, precipitates were weighed, and yields ranged from 8% to 10%. Since reaction temperature is an important factor that might affect protease activity, reactivity ratios were determined at both 25 and 40 °C. Table 2 shows that r_{Leu} and r_{Glu} reactivity ratios are all less than 1.0. Also, r_{Leu} and r_{Glu} are nearly identical for the different protease catalysts at a given reaction temperature. The only discernible trend is slightly lower values of r_{Leu} and r_{Glu} at 25 °C relative to 40 °C. That reactivity ratios are close to one

Table 2. Reactivity Ratio Values for L-$(Et)_2$-Glu/L-Et-Leu Oligomerizations Performed Using 1:1 Molar Feed Ratios and Papain, Bromelain, α-Chymotrypsin and Protease SG as Catalysts[a]

	reactivity ratios (r_{Leu} and r_{Glu})			
	25 °C		40 °C	
proteases	r_{Leu}	r_{Glu}	r_{Leu}	r_{Glu}
papain	0.79	0.76	0.89	0.94
bromalein	0.67	0.69	0.76	0.73
chymotrypsin	0.86	0.79	0.95	0.89
protease SG	0.75	0.68	0.80	0.78

[a] Reactivity ratios were determined by the Mayo−Lewis slope−intersection method. Co-oligomerization of L-$(Et)_2$-Glu and L-Et-Leu (total 2.5 mmol) catalyzed by different proteases (16 units/mL) in phosphate buffer (pH 8, concentration 0.9 M, 5 mL) for about 10−15 min at 25 and 40 °C. The resulting precipitate was immediately filtered, washed and analyzed.

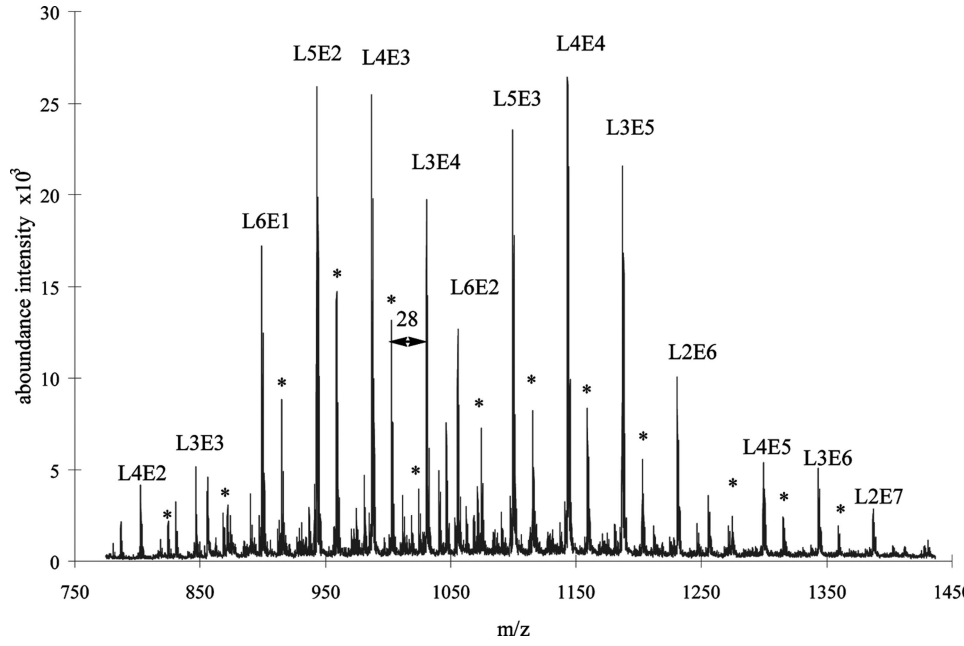


Figure 3. MALDI-TOF spectrum of oligo(γ-Et-L-Glu-*co*-L-Leu) synthesized using 0.25 M L-$(Et)_2$-Glu·HCl and 0.25 M L-Et-Leu·HCl, 16 units/mL papain, at 40 °C for 4 h, pH 8.5.

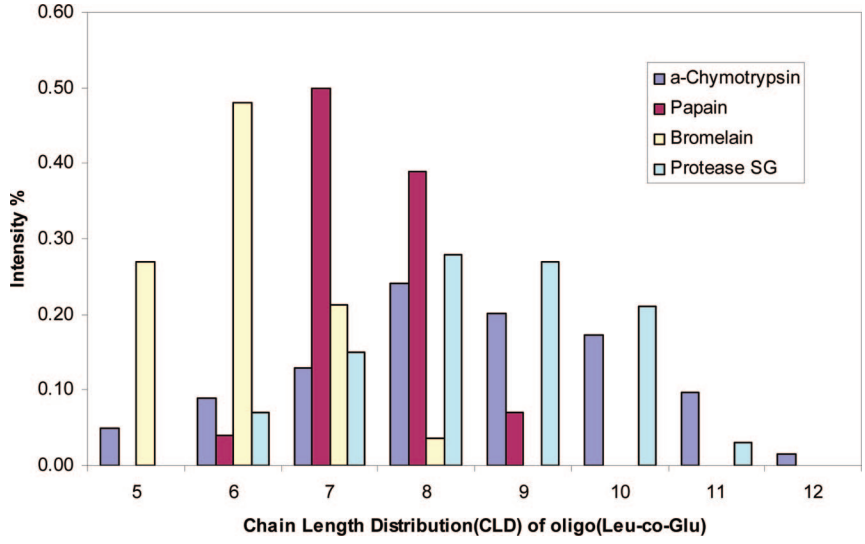


Figure 4. Chain length distribution (CLD) in MALDI-TOF spectra of oligo(γ-Et-L-Glu-*co*-L-Leu) synthesized using 0.25 M L$(Et)_2$-Glu·HCl and 0.25 M L-Et-Leu·HCl, 16 units/mL proteases, at 40 °C for 4 h, 0.9 M phosphate buffer, pH 8.5.

suggests that propagation during oligomer synthesis tends to occur randomly, giving random sequences along oligopeptides. Further study of oligopeptide sequence distribution by LC−MS and MALDI-TOF is discussed below.

Structural Analysis. The ^{1}H NMR spectrum of precipitated oligo(γ-Et-L-Glu-*co*-L-Leu), synthesized by papain-catalysis (40 °C, 4 h, feed ratio = 50:50, sample I, Table 1), along with peak positions and assignments, is displayed in Figure 2. Peak assignments in Figure 2 are predominantly based on those given in ref 19. Methine resonances of Leu and γ-Et-L-Glu repeat units (including those of C-terminal resonances) are found at 4.3 ppm (protons I), whereas N-terminal methine resonances for Leu and γ-Et-L-Glu repeat units are at ~3.6 ppm. Hence, direct comparison of signal intensities at 4.3 and 3.6 ppm was used to determine DP_{avg} values of co-oligopeptides. The relative content of γ-Et-L-Glu and L-Leu repeat units along co-oligopeptides was determined from relative intensities of signals corresponding to Leu methyl resonances A (0.9 ppm) and methylene protons F (2.3 ppm).

The MALDI-TOF spectrum displayed in Figure 3 is of the identical product characterized above by ^{1}H NMR. Two series of ion peaks, separated by a combined mass of 157 and 113 *m*/*z* units, equal to the mass of γ-Et-L-Glu and L-Leu repeat units, respectively, are observed, and peaks are isotopically resolved. Mass peaks corresponding to DP values 6−9 with various combinations of γ-Et-L-Glu and L-Leu units are observed. The most abundant signals correspond to co-oligopeptides with DP 7 and 8, in excellent agreement with DP_{avg} 7.3 determined by ^{1}H NMR spectroscopy (Table 1). Among all repeat unit combinations with DP 7, highest abundance intensity peaks correspond to co-oligopeptides with repeat unit compositions L5E2, L4E3 and L3E4. Similarly, for DP 8 co-oligopeptides, L5E3, L4E4 and L3E5 give peaks of highest intensities. Such distribution results indicate that papain, under these reaction conditions, has no preference or selectivity as to the addition of L-$(Et)_2$-Glu or L-Et-Leu to propagating oligomer chains. In other words, it appears that the co-oligomerization proceeded giving a random pattern of products. However, since

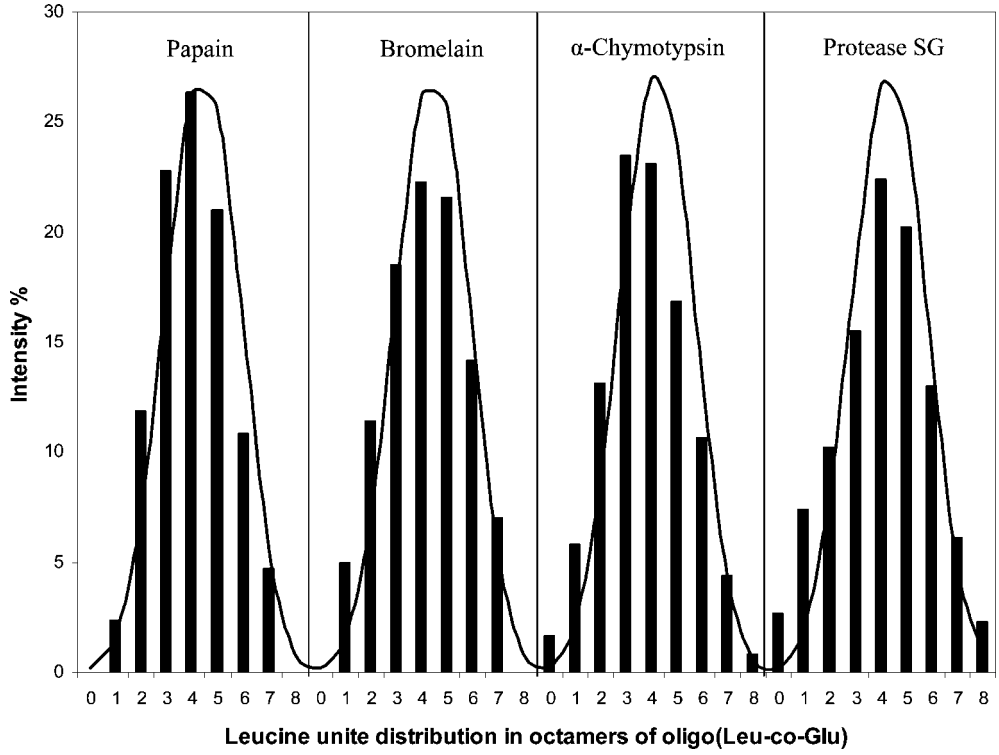


Figure 5. Leucine unit distribution in octamer from MALDI-TOF spectra of oligo(γ-Et-L-Glu-*co*-L-Leu) synthesized using 0.25 M L-$(Et)_2$-Glu·HCl and 0.25 M L-Et-Leu·HCl, 16 units/mL enzymes, at 40 °C for 4 h, 0.9 M phosphate buffer, pH 8.5. The curve is the theoretical distribution trend for each co-oligopeptide assuming a random distribution of repeat units.

Table 3. Papain Catalyzed L-$(Et)_2$-Glu/L-Et-Leu Co-Oligomerizations Performed Using a Broad Range of Monomer Feed Ratios[a]

feed ratio (mol) $[Leu]_0/[Glu]_0$	yield (mol)	DP[b]	product comp (mol) [Leu]/[Glu][b]	$[\alpha]_{589}$[c]	pH value after reaction
100/0	36	6.8	100/0	−35.9	5.7
90/10	46	6.4	89/11	−32.8	6.3
70/30	52	7.1	68/32	−24.2	6.0
50/50	68	7.3	52/48	−23.2	5.5
30/70	69	7.3	33/67	−19.6	6.1
10/90	72	7.6	12/88	−11.9	5.8
0/100	83	8.7	0/100	−8.7	6.1

[a] Co-oligomerization of L-$(Et)_2$-Glu and L-Et-Leu (total 2.5 mmol) catalyzed by papain (16 units/mL) in phosphate buffer (pH 8, concentration 0.9 M, 5 mL) at 40 °C for 4 h. [b] Determined by ^{1}H NMR. [c] Determined by polarimeter at $c = 0.01$, 25 °C in DMSO solvent.

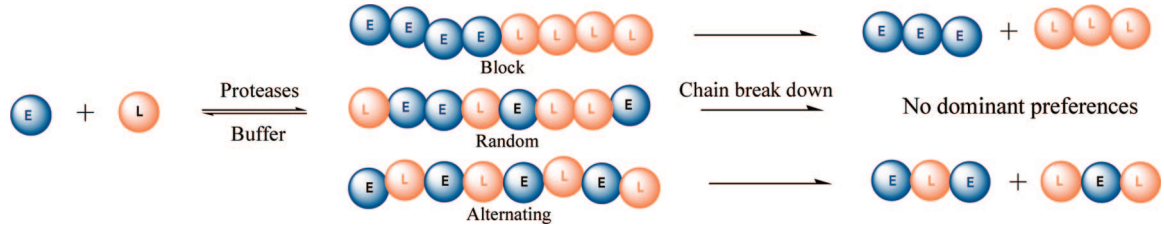
MALDI-TOF did not provide information on sequence, this analysis does not rule out the possibility that sequences along chains are block or alternating.

Given the complexity of the product mixtures formed with respect to both chain length and compositional distributions, the number of possible sequences is large. For example, for a co-oligopeptide with two different units and chain length of 8 units, there are 2^8 possible sequence combinations. This number of sequences does not lend itself to traditional sequencing techniques used for well-defined oligopeptide sequences prepared by traditional step-by-step solid state or solution peptide synthetic methods. In order to simplify the array of co-oligopeptide sequences, oligopeptides were cleaved so that the predominant chain length was 3 units. Then, the sequence distribution can be statistically analyzed by LC−MS that separates the eight possible standard (pure) 3-unit oligopeptides and quantitates 3-unit sequences obtained from protease-catalyzed oligomerizations. Details of methods used and results obtained from LC−MS analysis will be discussed below.

In addition to the cooligopetide produced by papain, further analysis of MALDI-TOF spectra of cooligopeptides (~50/50) catalyzed by the other three proteases (bromelain, α-chymotrypsin and protease SG) shows that the major series of signals are accompanied by a series of lower intensity peaks with *m*/*z* less by 28 (marked by an asterisk). This corresponds to hydrolysis of one ester group per oligo(γ-Et-L-Glu-*co*-L-Leu) chain that is located at either the C-terminal end or a γ-Et-L-Glu repeat unit along chains. No peaks were identified that correspond to hydrolysis of two or more ester moieties. An explanation for this observation is that enzyme-activated oligomer species can react with either the amino group of ethyl ester monomers or water (see Scheme 1). At high monomer conversion values reactions with water become increasingly prevalent, resulting in a fraction of chains with terminal carboxyl free acid groups. Thus, it is most likely that monocarboxyl oligomers have free acid groups at the α-carboxyl-C-terminal unit. Alternatively, oligopeptides can reach DP values of 7 to 8 and precipitate prior to activation by the protease. This will result in the observed fraction of oligomers that lack free carboxyl moieties. It is noteworthy that formation of two populations of oligopeptides consisting of monocarboxyl oligomers and oligomers lacking free acid groups was similarly observed by us for papain-catalyzed γ-Et-L-Glu homo-oligomerizations.[24]

Chain Length Distribution and Chemical Composition Analysis of Oligo(γ-Et-L-Glu-co-L-Leu) by MALDI-TOF. Co-oligomers described in Table 1, each synthesized using papain, bromelain, α-chymotrypsin or protease SG from a 1:1 mixture of L-$(Et)_2$-Glu/L-Et-Leu, were analyzed by MALDI-TOF spectroscopy. From MALDI-TOF spectra information was obtained on both the chain length distribution and chemical composition distribution. To our knowledge, there have been no previous investigations that applied MALDI-TOF to analysis of protease-catalyzed co-oligopeptides. The mass for single charged cooligomers were calculated and assigned according to refs 36 and

Scheme 2. Possible sequence selectivity of proteases during oligomerization reactions



37. Since another peak series consisting of mono-deesterified oligopeptides with the same chain length and chemical composition was also observed in the spectra, the total intensity ($I_{i,j}^{\text{total}}$) was adjusted by adding a deesterfied intensity ($I_{i,j}^{\text{deesterfied}}$) to the assigned highest intensity ($I_{i,j}$):

$$I_{i,j}^{\text{total}} = I_{i,j} + I_{i,j}^{\text{deesterfied}} \quad (1)$$

Figure 4 shows the total chain length distribution for oligo(γ-Et-L-Glu-*co*-~50mol%-L-Leu) products prepared using papain, bromelain, α-chymotrypsin or protease SG as catalyst. Based on the calculated total abundance intensity, the DP_{avg} values of these samples are 7.5, 6.1, 8.5 and 8.7 respectively. These values are in excellent agreement with DP_{avg} values for these samples calculated by ^{1}H NMR (7.3, 6.5, 8.2 and 8.5 respectively, Table 1). It is generally agreed that quantitative interpretations of MALDI-TOF results need to be interpreted with caution due to the possibility of discrimination effects resulting from mass differences and deviations in ionization efficiency resulting from variations in chemical composition.[38] Nevertheless, the outstanding agreement between DP_{avg} values from ^{1}H NMR and MALDI-TOF analyses improves our confidence in the quantitative value of oligo(γ-Et-L-Glu-*co*-~50mol%-L-Leu) MALDI-TOF results. Interestingly, Figure 4 shows differences in the breadth of oligomer chain length distributions as a function of the protease used. Oligopeptides synthesized using α-chymotrypsin and protease SG consist of the broadest chain length distributions, 5−12 and 6−11, respectively. In contrast, oligopeptides from bromelain and papain catalysis have chain length distributions of 5−8 and 6−9, respectively. These differences in chain length distribution are significant and are expected to influence the physical and biological properties of these and other oligopeptides synthesized by protease catalysis.

Figure 5 gives compositional distributions of Leu units in the octamer fraction for oligo(γ-Et-L-Glu-*co*-~50 mol %Leu)

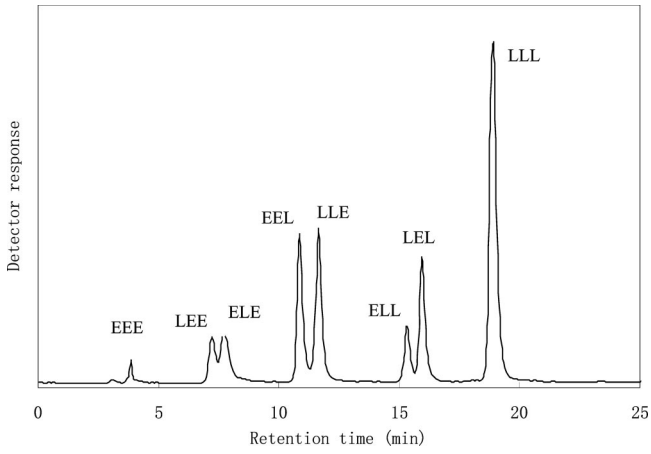


Figure 6. Triad elution profiles from HPLC; each sample was applied to a reversed-phase column (Polymer Laboratory PLRP-S) with dimensions 250 × 2.1 mm and particle size 5 μm. The mobile phase consisted of 10% H_2O, 85% acetonitrile, and 5% TFA. Data were collected and analyzed by MassLynx software.

samples prepared using papain, bromelain, α-chymotrypsin and protease SG as catalysts. Theoretical distribution trend curves for each of these co-oligopeptides are also shown in Figure 5. These curves were generated according to eq 2 assuming a random distribution of repeat units:

$$\text{possiblility} = (C_{\text{Leu}})^i (C_{\text{Glu}})^j N_{i,j} \quad (2)$$

where "possibility" is the possibility of each specimen, C is the percent composition calculated from NMR, i,j are the number of repeat units and $N_{i,j}$ is total number of specimens with identical repeat units (i and j).

Although MALDI-TOF can provide reliable average chemical composition measurements, results should be interpreted with caution due to the possibility of discrimination effects resulting from mass differences and deviations in ionization efficiency as discussed above. Results in Figure 5 indicate a large heterogeneity of leucine contents for oligomers synthesized using papain, bromelain, α-chymotrypsin and protease SG. In all cases, the highest abundance peaks appear in the range from 3 to 5 Leu units in octamers. This result is consistent with reactivity ratio values close to one suggesting that propagation during L-$(Et)_2$-Glu/L-Et-Leu co-oligomerizations occurs randomly giving oligopeptides with random sequences. Interestingly, MALDI-TOF spectra of co-oligopeptides prepared by papain and bromelain catalysts showed no peaks corresponding to the extreme compositions of Leu = 0 and 8. This result is in contrast to co-oligopeptides prepared using α-chymotrypsin and protease SG catalysts. These are small but significant differences in specificity between these two sets of proteases.

Papain Catalysis of L-$(Et)_2$-Glu/L-Et-Leu Co-Oligomerizations over a Broad Compositional Range. Papain catalyzed co-oligomerizations of L-$(Et)_2$-Glu and L-Et-Leu were performed with variation in monomer feed ratio, in phosphate buffer (pH 8.5, 0.9 M) at 40 °C for 4 h (Table 3). These reaction conditions were selected based on systematic studies by our laboratory to optimize L-$(Et)_2$-Glu homo-oligomerizations.[24] Since reaction conditions have not yet been optimized for L-Et-Leu oligomerizations, this may explain why the yield of oligo(γ-Et-L-Glu) is larger than that of oligo(L-Leu) (83 and 36%, respectively). As L-$(Et)_2$-Glu in the monomer feed ratio was increased from 0 to 50 mol %, the yield increased from 36 to 68%. However, further increases in the Lue/Glu ratio from 50:50 to 10:90 led to no significant changes in oligomer yield. DP_{avg} values of co-oligopeptides were approximately 7.0 throughout the compositional range, except for homo-oligo(γ-Et-L-Glu) whose DP_{avg} is 8.7. The general lack of dependence of DP_{avg} on co-oligopeptide composition can be explained by a correspondingly small effect of repeat unit composition on co-oligopeptide solubility. In other words, given that DP_{avg} is largely controlled by its solubility since isolated oligopeptide products are taken from the precipitated fraction of reactions, oligopeptide solubility shows little or no change across the compositions of oligopeptides prepared herein. Comparison of the comonomer feed ratios and corresponding oligomer repeat unit composition shows these values are in excellent agreement (Table 3). The final product

Table 4. LC−MS Analysis of Triad Hydrolysates Prepared from Oligo(γ-Et-L-Glu-*co*-~50mol%-L-Leu)

	papain (51:49)[a]		bromelain (52:48)[a]		α-chymotrypsin (49:51)[a]		protease SG (53:47)[a]	
	theor (mol %)	LC anal.[b]	theor (mol %)	LC anal.[b]	theor (mol %)	LC anal.[b]	theor (mol %)	LC anal.[b]
LLL	13	11 ± 3	14	12 ± 3	12	10 ± 3	15	14 ± 2
LLE	13	9 ± 4	13	11 ± 3	12	15 ± 3	13	11 ± 2
LEL	13	12 ± 4	13	8 ± 4	12	16 ± 4	13	9 ± 3
ELL	12	13 ± 2	13	15 ± 3	12	10 ± 3	13	15 ± 3
EEE	12	14 ± 2	11	18 ± 4	13	17 ± 4	10	12 ± 3
EEL	12	15 ± 4	12	11 ± 3	13	13 ± 3	13	13 ± 4
ELE	12	12 ± 3	12	14 ± 2	13	9 ± 3	13	11 ± 4
LEE	12	14 ± 3	12	11 ± 3	13	10 ± 1	13	15 ± 4

[a] Products were taken from an early stage precipitant (at <10% monomer conversion, 40 °C), ratio of γ-Et-L-Glu and L-Leu units determined by ^{1}H NMR
[b] Values reported are the mean from at least duplicate experiments, and the deviations defined the maximum and minimum values obtained.

specific rotation ($[\alpha]_{\S 89}$, $c = 0.01$) decreases as a function of leucine content in products (see column 4, Table 3).

During the course of L-$(Et)_2$-Glu/L-Et-Leu co-oligomerizations, one equivalent of HCl is liberated per addition of L-$(Et)_2$-Glu and L-Et-Leu monomers to propagating oligopeptide chains (see ref 24). Regardless that a high phosphate buffer concentration (0.9 M) was used to minimize pH decreases during reactions, measurements in reaction media at 4 h showed the pH decreased from an initial value of 8.5 to about 5.5−6.3 (column 5, Table 3). However, since previous work by our laboratory demonstrated that papain retains high activity (>80% relative to its maximum value) over the pH range from 5.5 to 8.5,[24] the observed pH decreases to ~5.5 should be tolerated by papain.

LC−MS Sequence Analysis. For protease catalyzed oligo(γ-Et-L-Glu-*co*-L-Leu) synthesis, products formed may be described by one of three extreme sequence distribution possibilities: block, random and alternating. Assuming that the sequence distribution is more complex than ideal block or random patterns, there can be up to 2^8 possible sequences for octamer chain lengths as discussed before. Hence, to simplify sequence analysis so that standards for possible sequences can be readily synthesized, chains were broken down chemically in order to maximize the population of triad sequences. At the trimer level, there are only 2^3 possible sequences that include EEE, LEE, ELE, EEL, LLE, ELL, LEL and LLL. All of these sequences were prepared chemically by standard solid-state Fmoc-based peptide chemistry using an automatic peptide synthesizer. Peptides were purified by reversed-phase HPLC on a semi-preparative column to give lyophilized white powders or crystals with purities >98% determined by HPLC. If the synthesized proteases tended toward a blocklike distribution, the predominant triads would consist of EEE and LLL sequences. Alternatively, if the synthesized proteases tended toward an alternating distribution of repeat units, the predominant triads would consist of ELE and LEL sequences. Random co-oligopeptides would give a more complex distribution of triad species (see Scheme 2). With purified triad sequences in hand, elution conditions were optimized using a reversed-phase column to separate triads according to their polarities on an LC−MS system. Ultimately, the eluent used was a mixture of water, acetonitrile and trifluoroacetic acid (10:85:5 v/v). LC−MS traces for standard samples are displayed in Figure 6. These show that the eight reference triads were successfully separated and the most polar and nonpolar triads, EEE and LLL, respectively, have the shortest and longest elution times, respectively (3.9 and 19.1 min). Standard UV response curves as a function of pure triad concentration, performed for each sequence possibility, allowed generation of standard curves for quantification. Subsequently, co-oligopeptide samples prepared from L-$(Et)_2$-Glu/L-Et-Leu (1:1 molar feed ratio) using the four proteases were prepared. Products were taken from an early stage precipitant (at <10% monomer conversion, 40 °C). This results in co-oligopeptides with compositions and sequence distributions that are determined based on enzyme specificity, instead of potentially being skewed by changes in the relative concentration of monomers as reactions proceed to higher monomer conversions. Co-oligopeptides were degraded to oligomers where the primary constituent in the population was trimers. This was accomplished by first removing ethyl esters under alkaline conditions (1 N NaOH, 60 °C, 36 h), neutralization with 6.0 M HCl, passing through a Sephadex G-10 column to desalt, and then performing hydrolysis in Pierce vacuum hydrolysis tubes at 110 °C for 60 min. Use of these harsh reaction conditions is known to show little or no preference for hydrolysis between specific Leu and Glu amino acids.[39] The hydrolysate with trimer constituents was then analyzed by LC−MS under the identical chromatographic conditions as were used for chemically synthesized standard triads. Tripeptides generated by oligopeptide hydrolysis were identified by comparison of elution time and mass with standard tripeptides, and quantification was accomplished using standard curves described above. The relative mol % of triads determined experimentally and by theoretical calculations which assumed a random statistical distribution is listed in Table 4. Comparison of experimental and theoretical values shows excellent agreement within the error of LC measurements. Therefore it is concluded that co-oligopeptides prepared by all four proteases have random distributions of repeat units.

Conclusion

The cumulative results of LC−MS, monomer relative reactivity ratios and MALDI-TOF determined compositional distribution curves lead to the conclusion that all four proteases showed no apparent specificity with respect to a preference for adding either L-Et-Leu to a Et-Glu terminal propagating chain, or L-$(Et)_2$-Glu to a Leu terminated chain. This results in the preparation of statistically random oligo(γ-Et-L-Glu-*co*-L-Leu) with $DP_{avg} \sim 7$ units. The compelling question raised by this work is which proteases will provide selectivity that regulates the sequence of this and other oligopeptides prepared from activated amino acid monomers in aqueous media. It may be that, presented with activated amino acid monomer pairs other than L-Et-Leu/L-$(Et)_2$-Glu, papain, bromelain, α-chymotrypsin and protease SG may show sequence specificity. Much work remains to provide answers to these important questions. The result of such efforts could lead to simple cost-effective routes to oligopeptides with various degrees of sequence control for a wide variety of applications.

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References and Notes

(1) Nowak, A.; Breedveld, V.; Pakstis, L.; Ozbas, B.; Pine, D.; Pochan, D.; Deming, T. *Nature* **2002**, *417*, 424–428.
(2) Whitesides, G. M.; Grzybowski, B. *Science* **2002**, *295*, 2418–2421.
(3) Lupo, M. P.; Cole, A. L. *Dermatol. Ther.* **2007**, *20*, 343–349.

(4) Langer, R.; Vacanti, J. P. *Science* **1993**, *260*, 920–926.
(5) Lu, Y.; Berry, S. M.; Pfister, T. D. *Chem. Rev.* **2001**, *101*, 3047–3080.
(6) Fairman, R.; Akerfeldt, K. *Curr. Opin. Struct. Biol.* **2005**, *15*, 453–463.
(7) Zhang, S.; Holmes, T.; Dipersio, M.; Hynes, R.; Su, X.; Rich, A. *Biomaterials* **1995**, *16*, 1385–1393.
(8) Vauthey, S.; Santoso, S.; Gong, H.; Watson, N.; Zhang, S. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5355–5360.
(9) Holmes, T. C.; de Lacalle, S.; Su, X.; Liu, G.; Rich, A.; Zhang, S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 6728–6733.
(10) Aggeli, A., B. M.; Boden, N.; Keen, J. N.; Knowles, P. F.; McLeish, T. C.; Pitkeathly, M.; Radford, S. E. *Nature* **1997**, *386*, 259–262.
(11) Pennington, M. W.; Dunn, B. M. *Peptide Synthesis Protocols*; Humana Press: Totowa, NJ, 1994; Vol. *35*.
(12) Iqbal Gill, R. L. p.-F.; Jorba, X.; Vulfson, E. N. *Enzyme Microb. Technol.* **1996**, *18*, 162–183.
(13) Hans-Dieter Jakubke, P. K.; Andreas, Konnecke *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 85.
(14) Aso, K. U., T.; Shiokawa, Y. *Agric. Biol. Chem.* **1988**, *52*, 2443–2449.
(15) Uemura, T. F., M.; Lee, H.-H.; Ikeda, S.; Aso, K. *Agric. Biol. Chem.* **1990**, 2277.
(16) Sluyterman, L. A. A. W., J. *Biochim. Biophys. Acta, Enzymol.* **1972**, *289*, 194–202.
(17) Gregory, A., P. L. L. *HeIv. Chim. Acta* **1979**, *62*, 488–494.
(18) Rolf Jost, E. B. J. C. M. P. L. L. *Helv. Chim. Acta* **1980**, *63*, 375–384.
(19) Uyama, H.; Fukuoka, T.; Komatsu, I.; Watanabe, T.; Kobayashi, S. *Biomacromolecules* **2002**, *3*, 318–323.
(20) Matsumura, S., Y. T.; Nobuyuki, Otozawa; Saeko, Murakami; Kazunobu, Toshima; Graham, Swift *Macromol. Rapid Commun.* **1999**, *20*, 7–11.
(21) Soeda, Y.; Toshima, K.; Matsumura, S. *Biomacromolecules* **2003**, *4*, 196–203.
(22) Eriksen, S.; Fagerson, I. S. *J. Food Sci.* **1976**, *41*, 490–493.
(23) Noar, S. R.; Shipe, W. F. *J. Food Sci.* **1984**, *49*, 1316–1317.
(24) Li, G.; Vaidya, A.; Viswanathan, K.; Cui, J.; Xie, W.; Gao, W.; Gross, R. A. *Macromolecules* **2006**, *39*, 7915–7921.
(25) Anson, M. L. *J. Gen. Physiol.* **1938**, *22*, 79–89.
(26) Bordusa, F. *Chem. Rev.* **2002**, *102*, 4817–4868.
(27) Bender, M. L.; Clement, G. E.; Gunter, C. R.; Kézdy, F. J. *J. Am. Chem. Soc.* **1964**, *86*, 3697.
(28) Twining, S. S. *Anal. Biochem.* **1984**, *143*, 30–34.
(29) Stoscheck, C. *Methods Enzymol.* **1990**, *182*, 50–69.
(30) Richardson, T.; Finley, J. W. *Chemical Changes in Food During Processing*; Springer: New York, 1985.
(31) Mitchel, R. E. *J. Biol. Chem.* **1970**, *245*, 3485–3492.
(32) Bonnie, C. S.-T. S. P. *Aquaculture* **2002**, *205*, 141–156.
(33) Glazer, A. N. *J. Biol. Chem.* **1966**, *241*, 635–638.
(34) Glazer, A. N. *J. Biol. Chem.* **1966**, *241*, 3811–3817.
(35) Gowariker V.R., V. N. V. J. S. *Polymer science*; New Age Publishers: New Delhi, 2005.
(36) Willemse, R. X. E.; Staal, B. B. P.; Donkers, E. H. D.; van Herk, A. M. *Macromolecules* **2004**, *37*, 5717–5723.
(37) Wilczek-Vera, G.; Danis, P. O.; Eisenberg, A. *Macromolecules* **1996**, *29*, 4036–4044.
(38) Wilczek-Vera, G. Y., Y.; Waddell, K.; Danis, O.; Eisenberg, A. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 764–777.
(39) Eveleigh, J. W. a. W., G.D. *Protein Sequence Determination: A Sourcebook of Methods and Techniques*; Springer-Verlag: New York, 1970.

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