Rapid Regioselective Oligomerization of L-Glutamic Acid Diethyl Ester Catalyzed by Papain

Geng Li, Alankar Vaidya, Kodandaraman Viswanathan, Junru Cui, Wenchun Xie, Wei Gao, and Richard A. Gross*

NSF I/UCRC for Biocatalysis and Bioprocessing of Macromolecules, Polytechnic University, Six Metro Tech Center, Brooklyn, New York 11201

Received June 23, 2006; Revised Manuscript Received August 11, 2006

ABSTRACT: Papain-catalyzed oligomerization of diethyl L-glutamate hydrochloride was conducted in phosphate buffer at 40 °C. Because of rapid oligomerization kinetics, high substrate concentrations were not needed to shift the equilibrium for oligomer synthesis. For example, at 0.03 M diethyl L-glutamate hydrochloride, oligo(γ-ethyl L-glutamate) synthesis and precipitation from solution occurred in 55% yield. MALDI-TOF spectra of precipitated products showed two series of ion peaks separated by 157 *m*/*z* units, the mass of oligo(γ-ethyl-L-glutamate) repeat units. The most abundant signals were at DP 8 and 9, in excellent agreement with DP_{avg} values determined by ^1H NMR. Lower intensity peaks with *m*/*z* less by 28 correspond to hydrolysis of one ester group either at a chain end or a pendant group along chains. Oligo(γ-ethyl-L-glutamate) synthesis at 40 °C in phosphate buffer (0.9 M, pH 7) occurred rapidly so that by 5, 10, and 20 min the yield reached 70 ± 4%, 78 ± 4% and 81 ± 5%, respectively. High product yields were observed over a broad range of pH values. As long as the pH was maintained from 5.5 to 8.5, the product yield was ≥60%. Ionic strength had no significant effect on oligopeptide yield. The dominant role of phosphate buffer in reactions was its control of pH. Other influences of phosphate ions on papain, such as nonspecific salt interactions or a "salting out" of product, appear to be of little or no importance. Loss in protein concentration and activity in the supernatant was observed after one reaction. A second reaction cycle performed using recovered supernatants resulted in a decrease in oligo(γ-ethyl-L-glutamate) yield from about 75% to 20%.

Introduction

Poly(amino acid)s are normally biodegradable in the environment. Furthermore, they may be biocompatible and provide biological activities that are useful in therapeutics. A commonly practiced route to synthesize poly(α-amino acid)s of high molecular weight is by ring-opening polymerization of α-amino acid *N*-carboxylic anhydrides (NCAs).[1,2] However, this involves toxic phosgene or their equivalents for monomer synthesis, and the reaction conditions require strict removal of water and high monomer purity. An alternative route is by thermal or acid-catalyzed polymerizations. Thermal condensation polymerization of aspartic acid followed by alkaline hydrolysis was used to prepare poly(aspartic acid). This product is water-soluble and biodegradable and is used as a metal chelator.[3] However, the harsh polymerization conditions result in racemization of aspartic acid and formation of chains with both α- and β-linked units. When poly(amino acid)s of defined stereochemistry and repeat unit composition are required, milder selective polymerization methods must be used. One such method is by protease-catalyzed oligopeptide synthesis.

Basic to protease catalyzed oligopeptide synthesis is equilibrium or thermodynamic control to direct reversal of proteolysis.[4−6] Difficulties encountered include low reaction rates, high stoichiometric amounts of enzyme, and the need to apply direct approaches to shift the reactions toward formation of desired products. Reaction conditions that lead to product precipitation or extraction increase efficiency of the reverse reaction. Kinetically controlled syntheses has proved useful for serine and cysteine proteases that form activated acyl enzyme intermediates during catalysis.[5] This approach generally involves use of activated acyl moieties, such as esters, as donor components which significantly accelerate the reaction rate. This study makes use of principles from both kinetic and thermodynamically controlled reactions in that reactants are activated by formation of esters and products precipitate from reactions.

Advantages of protease-mediated oligopeptide synthesis include (i) avoids racemization, (ii) decreased requirement for protection−deprotection steps, (iii) utilization of readily renewable and potentially inexpensive amino acid monomers, and (iv) mild nonhazardous operating conditions.[7,8] Aso et al.[9] reported oligomerization of dialkyl L-glutamate hydrochloride by protease catalysis. By increasing the organosolubility of papain by its modification with poly(ethylene glycol), Uemura et al.[10] showed oligomerizations of dialkyl L-aspartate and dialkyl L-glutamate can be performed in benzene to yield a mixture of oligomers 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 degree of polymerization (DP) less than 10.[11−13] Uyama et al.[14] reported protease-catalyzed regioselective polymerization and copolymerization of diethyl L-glutamate hydrochloride. By $^1H-^1H$ COSY NMR they showed that oligomers formed from diethyl L-glutamate hydrochloride using papain, bromelain, and α-chymotrypsin were exclusively α-linked. Matsumura et al.[15] reported that alkalophilic protease from *Streptomyces sp*. catalyzed the oligomerization of diethyl L-aspartate in bulk, forming a product with 88% α-linkages. Soeda et al.[16] found that diethyl L-aspartate was oligomerized by microbial protease BS in solutions containing small volumes of water. For example, oligomerizations of diethyl L-aspartate using protease BS were performed at 40 °C, for 2 days, in MeCN containing 4.5 vol % water. The resulting product was α-linked poly(β-ethyl L-aspartate) with M_w up to 3700 in 85% yield.

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

10.1021/ma061419y CCC: $33.50
Published on Web 10/13/2006

The above-cited publications demonstrate excellent progress has been made toward the synthesis of oligopeptides by protease catalysis. However, many of these studies did not take into account decreased pH during oligopeptide synthesis.[14,15] Reaction pH effects both catalyst activity and stability. Therefore, pH changes should be followed or eliminated by external addition of base during reactions. In addition, many of the above studies did not report catalyst activity on a standard substrate, making it impossible for others to reproduce their work.[9,14] Given the importance of developing general, facile, and selective routes to oligopeptides, our laboratory has begun studies to build on the above literature. However, given uncertainties from previous publications, this paper reports a reinvestigation of papain-catalyzed oligo(γ-ethyl-L-glutamate) synthesis under defined conditions. Activity of the papain catalyst was determined using a standard substrate (Ac−Phe−Gly−*p*-nitroanaline). Oligo(γ-ethyl-L-glutamate) synthesis was studied as a function of buffer pH and normality. Changes in solution pH during reactions were recorded. Papain activity as a function of pH was determined using pH control by automated or manual addition of sodium hydroxide. By performing reactions with controlled pH, we explored whether the buffer causes "salting out" of products or it is involved in nonspecific protein−ion interactions. Enzyme activity retained in the reaction media for multiple reaction cycles was assessed. The extraordinarily rapid formation of γ-ethyl-L-glutamate under controlled reaction conditions is reported.

Experimental Section

Materials. L-Glutamic acid diethyl ester hydrochloride and α-cyano-4-hydroxycinnamic acid (CCA) were purchased from Tokyo Kasei Co. Ltd. and Sigma, respectively, in the highest available purity and were used as received. Crude papain (EC # 3.4.22.2; source-*Carica papaya*; 30 000 USP units/mg of solid; molecular weight 21K) and chromogenic papain substrate Ac−Phe−Gly−*p*-nitroaniline (Ac−Phe−Gly−PNA) were 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. The protein content of the powder, determined by the bicinchoninic acid (BCA) method, is 40 wt %. Its activity was determined by the Ac−Phe−Gly pNA assay (see below).

Methods. General Procedure for Papain-Catalyzed Oligo(γ-ethyl-L-glutamate) Synthesis. L-Glutamic acid diethyl ester hydrochloride (600 mg, 2.5 mmol), papain (100 mg of water-soluble powder containing 40 mg protein), 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) and then twice with deionized water. The resulting product was lyophilized, giving a beige powder.

Controlling System pH. Since the pH decreases during oligomerizations, manual and automatic pH control was employed. Manual control was performed by using a VWR SympHony SB301 pH meter and manual addition of 10 M NaOH in response to observed pH changes. By manual control the reaction pH was maintained within ±0.1 pH units of the set value. Automatic controlled pH was performed using a Tiamo titration control system and Metrohm CH9101 dosing unit. The dosing solution (10 M NaOH) was added at 0.05−0.1 μL/min, and the frequency at which the probe checked the pH was set to 1.0 s. By automatic controlled pH with this hardware and software the pH was controlled within ±0.05 units of the set value.

Activity Assay. A stock solution was prepared consisting of a 1:1 (v/v) mixture of Ac−Phe−Gly−*p*-nitroaniline (Ac−Phe−Gly−PNA, 0.3 mg/mL) in DMF and 50 mM phosphate buffer pH 6.8. To 1 mL of substrate stock solution, 50 μL of papain (stock: 160 mg/mL protein) was added, and the absorbance was measured spectrophotometrically using a Shimadzu UV-1601 at 405 nm for 100 s. Enzyme activity per minute was calculated from the initial linear portion of the absorbance vs time curve. Activity units are the amount of enzyme activity which catalyzes the transformation of 1 μmol of the *p*-nitroaniline per minute in phosphate buffer (pH 6.8) at 25 °C using 8 mg/mL protein catalyst.

Instrumental Methods. Nuclear Magnetic Resonance (NMR) Spectroscopy. Proton (^{1}H) NMR spectra were recorded on a Bruker DPX 300 spectrometer at 300 MHz. NMR experiments were performed in DMSO-d_6 at 10 mg/mL with a data acquisition delay of 1s and a total of 64 scans. Data were collected and analyzed by MestRe-C. Proton chemical shifts were referenced to tetramethylsilane (TMS) at 0.00 ppm.

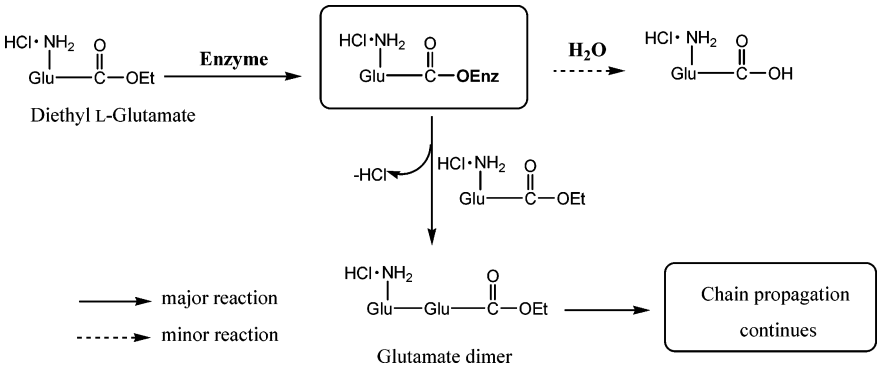
Mass-Assisted Laser Desorption/Ionization-Time-of-Flight (MALDI-TOF). MALDI-TOF spectra were obtained on an OmniFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Inc.). The instrument was operated in a positive ion linear mode with an accelerating potential of +20 kV. The TOF mass analyzer had pulsed ion extraction. The linear flight path was 120 cm. OmniFLEX TOF control software was used for hardware control and calibration. X-massOminFLEX 6.0.0 was used for data processing. 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 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.

Results and Discussion

For cysteine−protease-catalyzed peptide synthesis, the kinetic model mechanism (see Scheme 1)[5,17−19] 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 oligo(γ-ethyl-L-glutamate) from the reaction solution during synthesis further shifts the equilibrium toward peptide formation. Concurrent with peptide bond formation is release of HCl, which can lead to changes in medium pH during reactions. Papain prefers the α-position over γ of L-glutamate because of nature of its active site pocket. Uyama et al.[14] also reported the regioselective production of the oligoglutamate having an exclusive α-peptide linkage.

Effect of Substrate Concentration. Figure 1 shows the effect of substrate concentration on percent yield precipitated product for 20 min reactions under noncontrolled pH conditions (phosphate buffer 0.9 M). At initial pH 7, the percent yield increased from 56 ± 3% to 71 ± 4% by increasing the substrate concentration from 0.03 to 0.3 M. The yield remained unchanged as the substrate concentration was increased from 0.3 to 0.5 M and then decreased to 58 ± 3% by increasing the substrate concentration to 0.7 M. A similar trend was observed when reactions were performed at initial pH 8, except that product yields were higher than those at pH 7 for substrate concentrations 0.03−0.5 M. The apparent maximum percent yields (71 ± 4% and 81 ± 4%) occurred at 0.3 M substrate concentration for reactions at pH 7 and 8, respectively.

Scheme 1. Mechanism for Peptide Bond Formation (See Refs 5 and 17−19)



Scheme 1 shows that chain growth is accompanied by liberation of HCl from L-glutamic acid diethyl ester hydrochloride. Hence, by using initial pH 8 instead of 7, the reaction pH may remain within an optimum range of values where papain has higher activity for longer periods of time. This might explain relatively higher yields at pH 8 for substrate concentrations up to 0.4. However, as the substrate concentration increases at constant product yield, more HCl is liberated, creating the potential for decreased solution pH and lower yields. This may explain identical values of percent yield at pH 7 and 8 at high substrate concentrations (0.5−0.7). However, without knowledge of pH values during the course of reactions, it is difficult to provide definitive explanations. Substrate concentration is not an independent variable since higher substrate concentrations can lead to larger changes in solution pH that effects enzyme activity. These results, where explanations required knowledge of pH change, motivated the studies below where pH was either monitored or controlled during reactions.

Figure 1 showed that, regardless of pH change, oligomerizations at initial pH 7 and 8 occur over a broad range of substrate concentrations with yields greater than 50%. Thus, kinetic constants for chain propagation forming oligomers that precipitate from solution must be large. Rapid oligomerization kinetics allows formation of product without relying on high substrate concentration to shift the equilibrium.

Structural Analysis. The ^{1}H NMR spectrum of the precipitated product synthesized by papain catalysis (8 mg/mL catalyst, 0.9 M phosphate buffer, at 40 °C, for 20 min under noncontrolled pH conditions) is displayed in Figure 2. Peaks positions and assignments are identical to those described in ref 14 for oligo(γ-ethyl-L-glutamate). Relative integration of methane proton signals F and D (4.3 and 3.8 ppm, respectively) was used to calculate DP_{avg} for oligo(γ-ethyl-L-glutamate)s. The DP_{avg}, 8.7 ± 0.3, varied little for oligo(γ-ethyl-L-glutamate) synthesized herein over a wide range of reaction conditions. 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 157 *m*/*z* units, equal to the mass of oligo(γ-ethyl-L-glutamate) repeat units, are observed and the peaks are isotopically resolved. Mass peaks corresponding to DP values 7−11 are seen in Figure 3. The most abundant signals were at DP 8 and 9, in excellent agreement with DP_{avg} values determined by ^{1}H NMR. The major series of signals with *m*/*z* 1169, 1326, 1483, 1640, and 1797 was accompanied by a series of lower intensity peaks with *m*/*z* less by 28. This corresponds to hydrolysis of one ester group either at a chain end or a pendant group along oligo(γ-ethyl-L-glutamate). Thus, results of MALDI-TOF suggest that the product is a mixture of completely esterified and monocarboxylic acid oligomers. A route by which amide formation is the major pathway accompanied by less frequent hydrolysis is depicted in Scheme 1.

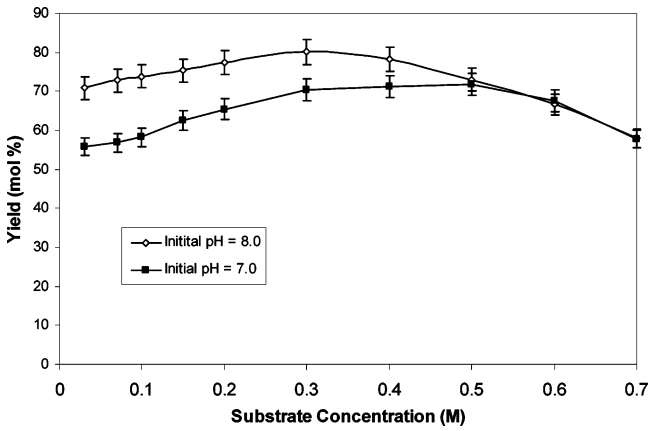


Figure 1. Effect of substrate concentration on percent yield of oligo(γ-ethyl-L-glutamate). Reactions were conducted with 8 mg/mL catalyst, 0.9 M phosphate buffer, at 40 °C, for 20 min, under noncontrolled pH conditions. Values reported are the mean from at least duplicate experiments, and error bars define the maximum and minimum values obtained.

Aso et al.[9] and Uyama et al.[14] found that crude papain extracts catalyzed oligomerizations of L-glutamic acid diethyl ester (conditions: pH 7−8.5, 0.5 M, ionic strength 2.0 M phosphate buffer, 25 °C and pH 7.0, 40 °C, 3 h) giving oligomers with DP 5−9 and DP_{avg} 9.5 from FAB−mass spectroscopy and ^{1}H NMR analysis, respectively. Hence, the molecular weights of products formed in this and related published work are in good agreement. This is largely due to that molecular weights of products are determined by their solubility in the reaction medium. In other words, when chains grow to a length at which they are poorly soluble, they precipitate from solution.

Effect of Enzyme Concentration. Figure 4 displays the effect of papain concentration on percent yield. L-Glutamic acid diethyl ester hydrochloride (0.5 M) oligomerizations were conducted in 0.9 M phosphate buffer at pH 7.0 for 20 min under noncontrolled pH conditions. By increasing the catalyst concentration from 2.0 to 2.8 to 4.0 mg/mL (based on the protein content of the powder), the percent yield increased from 29 ± 2% to 53 ± 2% to 74 ± 4%, respectively. Further increase in enzyme concentration resulted in no significant change in product yield. From the above, 8 mg/mL catalyst was used for other experiments described below. Uyama et al.[14] reported that, with 0.5 M L-glutamic acid diethyl ester in phosphate/sodium chloride buffer (pH 7.0, I = 2.0 M), maximum yields (61%) were obtained after 3 h using 40 mg/mL papain. Thus, the reaction was performed 9 times longer and required a much higher papain concentration. This may be explained by lower purity and/or activity of the papain preparation that was used.

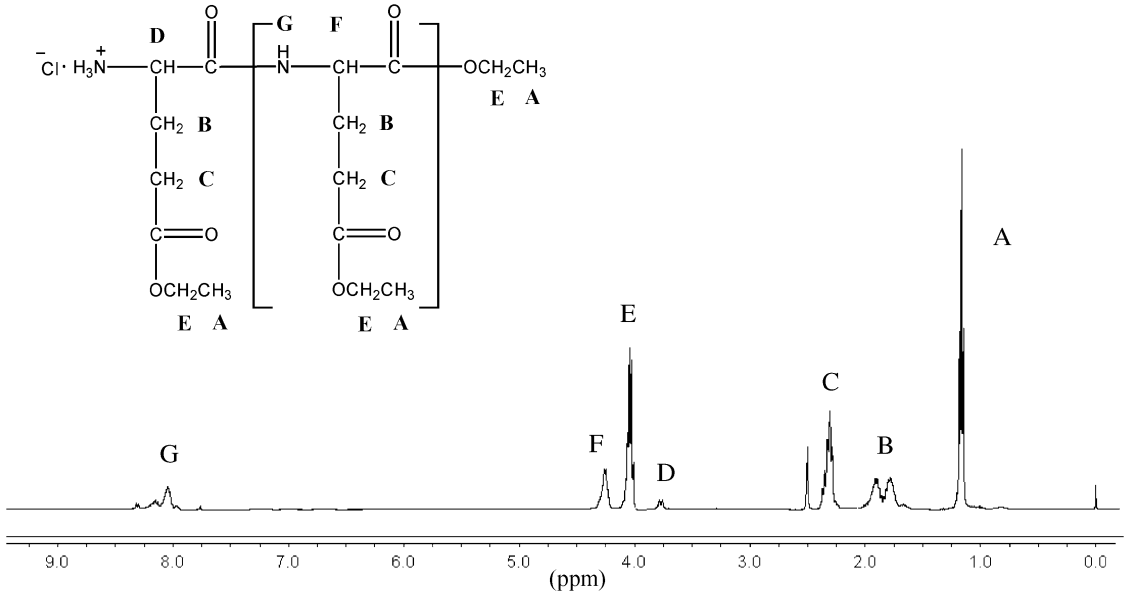


Figure 2. ^{1}H NMR (300 MHz, DMSO-d_6) spectrum of oligo(γ-ethyl-L-glutamate) synthesized using 0.5 M L-glutamic acid diethyl ester hydrochloride, 8 mg/mL catalyst, 0.9 M phosphate buffer, at 40 °C, for 20 min, under noncontrolled pH conditions.

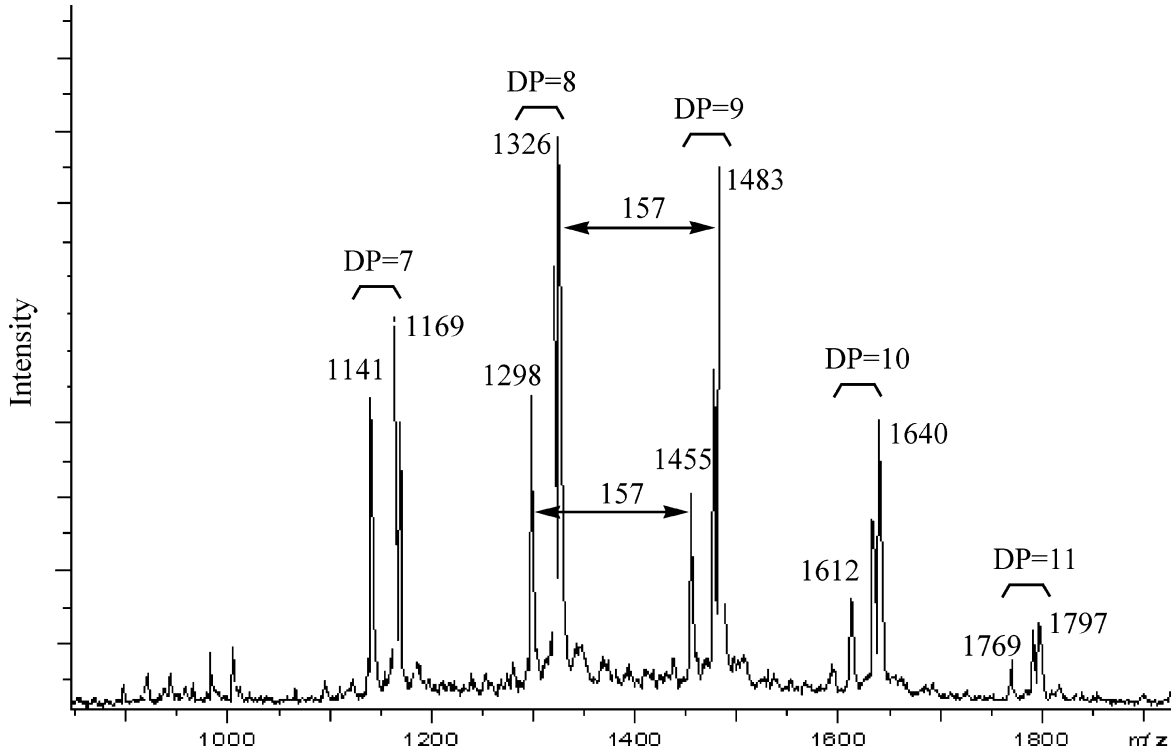


Figure 3. MALDI-TOF spectrum of oligo(γ-ethyl-L-glutamate) synthesized using 0.5 M L-glutamic acid diethyl ester hydrochloride, 8 mg/mL catalyst, at 40 °C for 20 min, pH 7.0, under noncontrolled pH conditions.

However, Uyama et al.[14] did not report papain activity, making it difficult to interpret differences observed.

Time Course of Oligo-L-(glutamic acid ester) Synthesis. To investigate the time course of oligo(γ-ethyl-L-glutamate) synthesis, 8 mg/mL papain catalyst and 0.5 M substrate were incubated at 40 °C in phosphate buffer (0.9 M, pH 7). No induction period was required, and by 5, 10, and 20 min, the yield reached 70%, 78%, and 81 ± 5%, respectively (Figure 5). Further increase in the reaction time beyond 20 min did not significantly increase product yield. Hence, under these conditions, the oligomerization is rapid, making this reaction attractive from both fundamental and commercial viewpoints. Investigation of reaction progress at short time intervals (≤20 min) is crucial to accurately assess effects of reaction parameters on product yield. However, previous work used longer reaction times for such studies. For example, Uyama et al.[14] used higher concentrations of papain (40 mg/mL) than this study and 3 h reaction times to assess effects of monomer concentration, enzyme concentration, and ionic strength on oligo(γ-ethyl glutamate) yield. Similarly, even though Aso et al.[9] reported high oligomer yield after 1 h, they assessed effects of substrate concentration, reaction time and buffering agent for 3 or 24 h reactions. In these cases, it may be that differences in polymer yields due to changes in reaction parameters are blurred since time is given so that slow reactions can "catch up" to those under more optimal conditions.

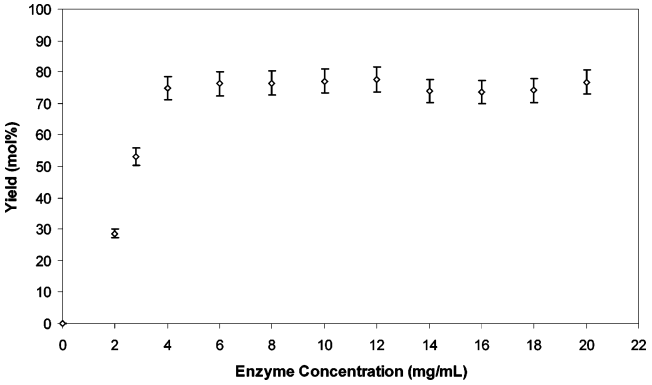


Figure 4. Effect of enzyme concentration on percent yield of oligo(γ-ethyl-L-glutamate). Reactions were conducted using L-glutamic acid diethyl ester hydrochloride (0.5 M) as monomer in 0.9 M phosphate buffer pH 7.0 for 20 min under noncontrolled pH conditions. Values reported are the mean from at least duplicate experiments, and error bars define the maximum and minimum values obtained.

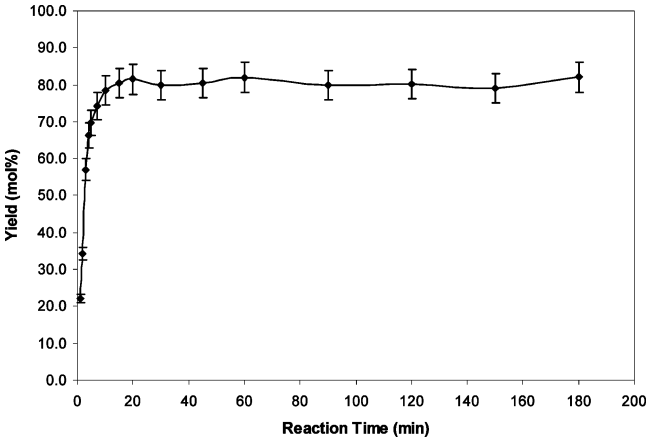


Figure 5. Time course of oligo(γ-ethyl-L-glutamate) synthesis. Reactions were conducted with 8 mg/mL papain catalyst, 0.5 M L-glutamic acid diethyl ester hydrochloride, at 40 °C, in phosphate buffer (0.9 M, pH 7) under noncontrolled pH conditions. Values reported are the mean from at least duplicate experiments, and error bars define the maximum and minimum values obtained.

The possibility that products may be hydrolyzed by extended time exposure to protease was studied. Products obtained at 20 min and 3 h were both analyzed by MALDI-TOF and ^{1}H NMR. However, no observable difference in percent hydrolysis of ester side chains was found for these products. This is explained by precipitation from solution of oligo(γ-ethyl glutamate) limiting its accessibility to protease.

Interplay between pH, Buffer Concentration, and Ionic Strength. Biotransformations should be performed within the optimum activity range of the enzyme catalyst. In the present system, pH decreases as the reaction progresses due to HCl liberation. One method to control pH change is by adjusting the buffer strength. However, increasing the buffer concentration also increases salt concentration, which might alter enzyme activity. Furthermore, after attaining a certain molecular weight, the product precipitates from solution. The salt concentration in reactions may change product yield and molecular weight by lowering the products solubility in the reaction medium (i.e., "salting out" effect). This complex set of variables was systematically studied as described below.

Oligomerizations were performed as a function of reaction time and phosphate buffer concentration. Figure 6 shows that, at 1.3 M phosphate buffer, the reaction pH decreased from 7.0 to 5.9 in just 20 min. By further reducing the phosphate buffer

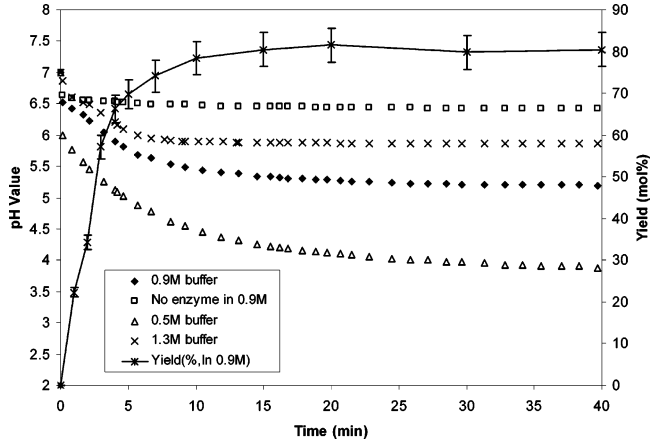


Figure 6. Change in pH during oligo(γ-ethyl-L-glutamate) synthesis for the control (0.9 M) and reactions in 0.5, 0.9, and 1.3 M phosphate buffer. Time course of oligo(γ-ethyl-L-glutamate) synthesis was performed at 40 °C in 0.9 M phosphate buffer at pH 7 under noncontrolled pH conditions. Values reported are the mean from at least duplicate experiments, and error bars define the maximum and minimum values obtained.

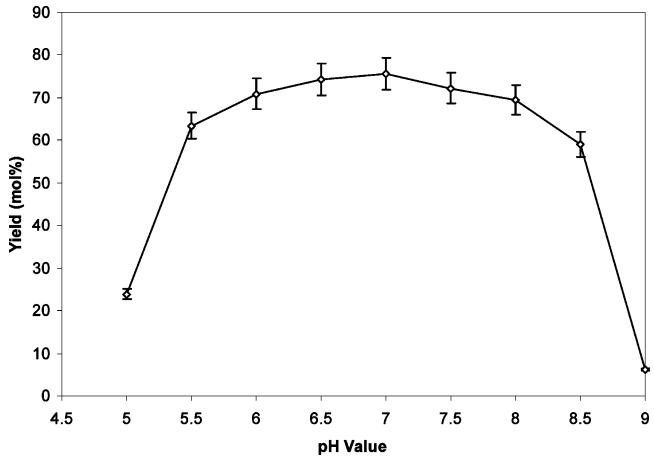


Figure 7. Relationship between reaction pH and oligo(γ-ethyl-L-glutamate) yield. Reactions were conducted using an automated pH-stat, 0.9 M phosphate buffer, 0.5 M L-glutamic acid diethyl ester hydrochloride, 8 mg/mL catalyst, at 40 °C for 20 min. Values reported are the mean from at least duplicate experiments, and error bars define the maximum and minimum values obtained.

concentration, pH values in reactions decreased to larger extents. For example, at 0.5 M phosphate buffer, the reaction pH decreased from 7.0 to 4.1 in 20 min. Even using a moderately high phosphate buffer concentration (0.9 M), decrease in pH by 20 min is substantial (from 7 to 5.3). A no-enzyme control, performed at 0.9 M phosphate buffer, showed no change in reaction pH to greater than 40 min. Excellent correlation was found between oligomer formation and pH decrease over time. For example, at 0.9 M phosphate buffer (initial pH 7.0), the pH decreases as oligomer formation increases to 15 min, after which neither showed any further change.

The relationship between reaction pH and product yield was studied to establish a suitable pH range at which papain-catalyzed oligo(γ-ethyl-L-glutamate) synthesis can be performed (Figure 7). The reaction pH was maintained at different pH values (5.0−9.0) using an automated pH-stat (see Experimental Section). Reactions were performed as follows: 0.5 M L-glutamic acid diethyl ester hydrochloride monomer, 8 mg/mL catalyst, 40 °C, 20 min. High product yields were observed over a broad pH range. As long as the pH was maintained at from 5.5 to 8.5, the product yield was ≥60%. Maximum product

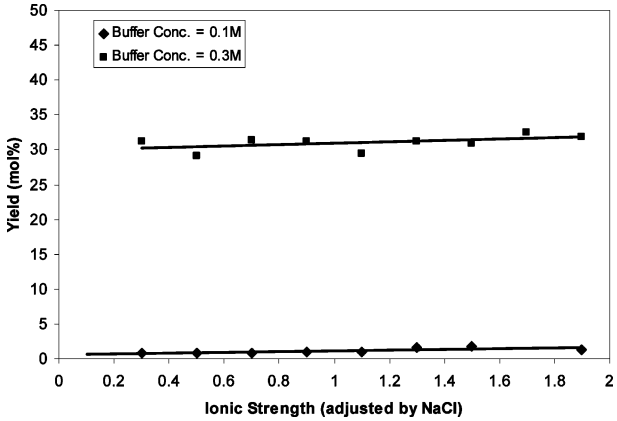


Figure 8. Effect of ionic strength (*I*) on oligo(γ-ethyl-L-glutamate) yield. The ionic strength of each buffer was adjusted to 1.9 M by addition of NaCl. Reactions were performed with 0.5 M L-glutamic acid diethyl ester hydrochloride, 8 mg/mL catalyst, at 40 °C for 20 min, without external pH control.

yields between 70 and 75% were reached by maintaining the pH between 6 and 8. Product yields decreased to 63 ± 3% and 24 ± 1% by performing reactions at pH 5.5 and 5, respectively. Similarly, product yields decreased to 59 ± 3% and 6.8 ± 0.5% by performing reactions at pH 8.5 and 9, respectively. Therefore, care must be taken to ensure that the reaction pH does not fall below 5.5 or above 8.5.

To study the effect of ionic strength (*I*) on product yield (Figure 8), phosphate buffer (pH 7.0) was prepared in which the phosphate ion concentration of buffer was maintained at 0.1 and 0.3 M. The ionic strength of each buffer was then adjusted to 1.9 M by addition of NaCl. Reactions were performed as above with 0.5 M L-glutamic acid diethyl ester hydrochloride, 8 mg/mL catalyst, at 40 °C for 20 min; however, pH of reactions was not controlled. Results in Figure 8 show that the ionic strength had no significant effect on yields of precipitated oligopeptide. Furthermore, DP_{avg}, calculated from ^{1}H NMR, was found to be independent of ion strength. At 0.3 M phosphate buffer, product yield was unchanged irrespective of the NaCl concentration. Furthermore, the rapid decrease of pH for reactions run at 0.1 M resulted in little product yield for ionic strengths ranging from 0.1 to 1.9 M. Thus, it is not ionic strength (adjusted by NaCl) that determines oligomer yield but, instead, it is the capacity of regulating system pH that is crucial. In contrast, Uyama et al.[14] reported that buffer ionic strength (pH 7.0) plays a decisive role in determining the yield of the same oligomerization reaction. In addition, they did not consider the role of pH change on the oligomerization.

To further clarify relationships between phosphate concentration, pH change, and oligomers yield as well as to investigate phosphate buffer concentration as an independent variable, experiments were performed at varying phosphate buffer concentrations with and without external pH control. Reactions were performed with 0.5 M L-glutamic acid diethyl ester hydrochloride, 8 mg/mL protein catalyst, 40 °C, pH 7.0 for 20 min. Without external pH control, the percent yield oligomers increased from about 4% to 82% as phosphate buffer concentration was increased from 0.1 to 1.1 M (Figure 9). Results above showed that, by maintaining the pH at between 5.5 and 8.5, the product yield was ≥60% (see Figure 7).

However, papain activity decreased when the pH dropped to 5.0. Without pH control, using 0.9 M phosphate buffer, the pH remained at ≥5.5 to 10 min and gave product yields at 10 min of 79% (see Figure 6). It follows that nearly optimal yields were attained without pH control by using 0.9 M phosphate buffer.

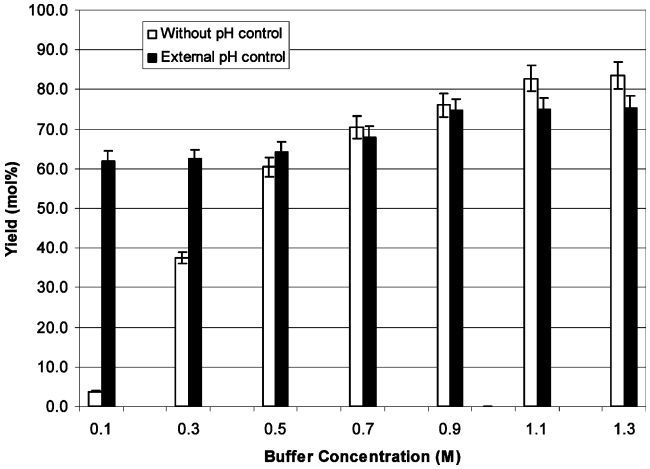


Figure 9. Effect of phosphate buffer concentration on oligo(γ-ethyl-L-glutamate) yield. Reactions were performed with 0.5 M L-glutamic acid diethyl ester hydrochloride, 8 mg/mL catalyst, 40 °C, pH 7.0 for 20 min with and without external pH control. Values reported are the mean from at least duplicate experiments, and error bars define the maximum and minimum values obtained.

At phosphate buffer concentrations >0.9 M (i.e., 1.3 M, see Figure 6), reaction pH remains above 5.5, papain activity remains high, and product yields are optimal. In contrast, by decreasing phosphate buffer concentration from 0.9 to 0.5 M, oligomer yield decreased from 76% to 60%. This decrease in oligomer yield is due to the relatively poor buffering capacity of 0.5 M phosphate buffer (Figure 6). Hence, by performing reactions with 0.3 and 0.1 M phosphate buffer, oligo(γ-ethyl-L-glutamate) yield decreased to 37.5 ± 3.0% and 3.8 ± 2.0%, respectively.

Increasing the buffer concentration also increases the phosphate content in reactions which might alter enzyme activity. Also, after attaining a certain molecular weight (see below), oligo(γ-ethyl-L-glutamate) precipitates from solution. By increasing the phosphate salt concentration oligo(γ-ethyl-L-glutamate), the yield might increase due to "salting out" of product. To investigate these possibilities, reactions were carried out with controlled pH at different buffer concentrations (Figure 9). Without pH control, at 0.1 and 0.3 M phosphate buffer concentrations, product yields were quite low: 3.8 ± 2.0% and 37.5 ± 3.0%, respectively. However, by externally controlling the pH at 7.0, product yield at these low buffer concentrations increased to about 62%. This proves that high product yields can also be achieved at low phosphate buffer concentrations as long as the pH of the reaction is controlled. At phosphate buffer concentrations ≥0.5 M, differences between percent product yield with and without pH control are small. Therefore, the dominant role of phosphate buffer in reactions was its control of pH. Other influences of phosphate ions on papain, such as nonspecific salt interactions or a "salting out" of product, appear to be unimportant.

The high sensitivity of papain to pH changes in reactions is consistent with what is known about enzyme structure and mechanism of action. The catalytic activity of papain is based on a nucleophilic attack of the cystein residue, and the activity requires two ionizable residues: Cys-25 and His-159. Only near neutral pH do these key amino acids have the appropriate ionization state that favors synthesis.[20] At low or acidic pH values, His-159, which must be in the deprotonated state for acyl−enzyme complex formation, remains protonated, leading to low enzyme activity. In contrast, at high or alkaline pH values, a proton is abstracted from Cys-25, destabilizing the intermediate transition state of the acyl−enzyme complex.

Table 1. Papain in the Supernatant for Catalysis of a Second Reaction Cycle

	total protein concn[a] (μg/mL)		specific activity × 10^{-3} units[b]		product yield (mol %)	
	before	after	before	after	cycle 1	cycle 2
0.9 M	7693	1064	0.89	0.11	76.1 ± 3.0	20.9 ± 2.0
0.9 M (maintaining pH)	7273	2023	0.94	0.31	74.6 ± 3.0	20.2 ± 2.0

[a] Protein was measured by the bicinchoninic acid (BCA) method, and papain protease fraction activity was measured using the chromogenic substrate Ac−Phe−Gly−PNA (see Experimental Section). [b] Unit is defined as the amount of enzyme activity which catalyzes the transformation of 1 μmol of *p*-nitroaniline per minute at pH 6.8 phosphate buffer and 25 °C using 8 mg/mL protein catalyst.

Potential of System for Multiple Reaction Cycles. Work above demonstrated that oligo(γ-ethyl-L-glutamate) is synthesized in high yields within 20 min. This learning motivated us to explore whether papain remaining in the reaction medium after one reaction could be used for a second oligomerization reaction. The total protein concentration (TPC) and specific activity were determined before and after reactions performed in 0.9 M buffer with and without external pH control (see Table 1). As above, reactions were carried out with 0.5 M L-glutamic acid diethyl ester monomer at 40 °C for 20 min. Table 1 shows that, after one reaction cycle, the TPC and specific activity for the reaction without external pH control decreased by 86% and 88%, respectively. After one reaction cycle with external pH control, the TPC and specific activity decreased by 72% and 67%, respectively. Hence, there is good agreement between percent decreases in TPC and specific activity. Also, pH control decreased loses in both TPC and specific activity after one reaction cycle. In both cases, proteins in the papain extract coprecipitate with oligo(γ-ethyl-L-glutamate). These proteins were removed from oligo(γ-ethyl-L-glutamate) by washing the precipitate with copious amounts of deionized water. A second reaction cycle was performed using recovered supernatants characterized above. As above, reactions were conducted for 20 min with and without external pH control. In both cases, the percent yield of oligo(γ-ethyl-L-glutamate) decreased from about 75% to 20%. The larger loss in activity for the non-pH-controlled reaction predicted by the Ac−Phe−Gly−PNA assay was not observed for oligo(γ-ethyl-L-glutamate) synthesis. Methods to improve papain stability by conjugation to carbohydrates[21] and poly(ethylene glycol)[9,21] were previously reported. Future work will consider using these strategies, as well as others such as immobilization onto solid supports, to improve papain stability so that it may be reused for multiple reaction cycles.

Conclusion

Papain-catalyzed oligomerization of diethyl L-glutamate hydrochloride was conducted in 0.9 M phosphate buffer at 40 °C to give oligo(γ-ethyl-L-glutamate) in 80% yield by 10 min. Concurrent with peptide bond formation is release of HCl. Changes in medium pH must be managed as this can affect enzyme activity. Nearly optimal yields were obtained without pH control at 0.9 M phosphate buffer. Decrease in phosphate buffer concentration from 0.9 to 0.5 M resulted in a decrease in oligomer yield from 76% to 60%. This is explained by decrease in reaction pH below 5.0 within 5 min. Values of pH below 5.5 result in decreased papain activity for oligo(γ-ethyl-L-glutamate) synthesis. The dominant role of phosphate buffer in reactions was its control of pH. Other influences of phosphate on papain such as nonspecific salt interactions, or a "salting out" of product, appear to be of little or no importance. The predominant species in the molecular weight distribution by MALDI-TOF analysis are DP 8 and 9. This agrees with DP_{avg} calculations from ^1H NMR. Analysis of reaction supernatant after product formation and precipitation showed large losses in both protein concentration and enzyme activity. Recycling of the reaction supernatant resulted in a decrease in oligo(γ-ethyl-L-glutamate) yield from 75% to 20%.

Acknowledgment. We are grateful to the NSF-I/UCRC for Biocatalysis and Bioprocessing of Macromolecules and its industrial members (BASF, DNA 2.0, Johnson & Johnson, Rohm and Haas, Genencor, Estee Lauder, Novozymes, W.R.Grace) for financial support and intellectual input.

References and Notes

(1) Klok, H.-A.; Lecommandoux, S. *Adv. Mater.* **2001**, *13*, 1217.
(2) Gallot, B. *Prog. Polym. Sci.* **1996**, *21*, 1035.
(3) Swift, G. *Polym. Degrad. Stab.* **1998**, *59*, 19.
(4) Rozzell, J. D., Wagner, F., Eds.; *Biocatalytic Production of Amino Acids and Derivatives*; Hanser Publishers: Muenchen, 1992.
(5) Bordusa, F. *Chem. Rev.* **2002**, *102*, 4817.
(6) Hans-Dieter Jakubke, P. K.; Konnecke, A. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 85.
(7) Gill, I.; Lopez-Fandino, R.; Jorba, X.; Vulfson, E. N. *Enzyme Microbial Technol.* **1996**, *18*, 162.
(8) Tepanov, V. M. S. *Pure Appl. Chem.* **1996**, *68*, 1335.
(9) Aso, K. U. T.; Shiokawa, Y. *Agric. Biol. Chem.* **1988**, *52*, 2443.
(10) Uemura, T. F. M.; Lee, H.-H.; Ikeda, S.; Aso, K. *Agric. Biol. Chem.* **1990**, *54*, 2277.
(11) Sluyterman, L. A. A.; Wijdenes, J. *Biochim. Biophys. Acta* **1972**, *289*, 194.
(12) Anderson, G.; Luisi, P. L. *Helv. Chim. Acta* **1979**, *62*, 488.
(13) Jost, R.; Brambilla, E.; Monti, J. C.; Luisi, P. L. *Helv. Chim. Acta* **1980**, *63*, 375.
(14) Uyama, H.; Fukuoka, T.; Komatsu, I.; Watanabe, T.; Kobayashi, S. *Biomacromolecules* **2002**, *3*, 318.
(15) Shuichi, M.; Yasuhiro, T.; Nobuyuki, O. *Macromol. Rapid Commun.* **1999**, *20*, 7.
(16) Soeda, Y.; Toshima, K.; Matsumura, S. *Biomacromolecules* **2003**, *4*, 196.
(17) Bender, M. L.; Clement, G. E.; Gunter, C. R.; Kezdy, F. J. *J. Am. Chem. Soc.* **1964**, *86*, 3697.
(18) Fastrez, F.; Fersht, A. R. *Biochemistry* **1973**, *12*, 2025.
(19) Jakubke, H.-D. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 175.
(20) Lewis, S. D.; Johnson, F. A.; Shafer, J. A. *Biochemistry* **1976**, *15*, 5009.
(21) Wartchow, C. A.; Wang, P.; Bednarshki, M. D.; Callstrom, M. R. *J. Org. Chem.* **1995**, *60*, 2216.

MA061419Y