ACS POLYMERS Au
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Chemoenzymatic Synthesis of Poly-L-lysine via Esterification with Alcohol in One-Pot

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Cite This: ACS Polym. Au 2025, 5, 26−34

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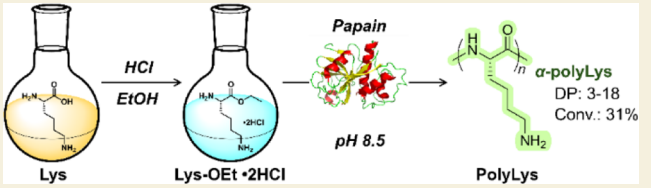
ABSTRACT: Chemoenzymatic polymerization (CEP) using enzymes as catalysts is gaining attention as an environmentally friendly method for synthesizing polypeptides. This method proceeds under mild conditions in aqueous solvents and leverages the substrate specificity of enzymes, allowing polymerization reactions to occur without the need to protect reactive side-chain functional groups. However, the monomers used must have esterified C-termini, such as amino acids or oligopeptides. In this study, we used L-lysine (Lys-OH) as a model example and performed one-pot CEP with papain without isolating the esterified lysine. Esterification of Lys-OH was achieved by using hydrochloric acid as a catalyst in ethanol, and one-pot polymerization resulted in poly-L-lysine (polyLys) with a peak top degree of polymerization (DP) of 6 and a maximum DP of 18, with a 31% conversion from the nonesterified lysine. The obtained polyLys was all α-linked, demonstrating that regioselective polymerization was successfully achieved even with one-pot CEP.



KEYWORDS: *chemoenzymatic polymerization, esterification, papain, polypeptide, one-pot synthesis*

INTRODUCTION

Polypeptides and proteins, which constitute a class of biopolymers, have emerged as versatile and promising materials for structural and functional applications. Owing to their unique combination of biocompatibility, tunable mechanical properties, and diverse functionality, polypeptides are excellent candidates for a wide range of applications.[1−4] The intrinsic modular nature of polypeptide sequences enables precise manipulation of their properties, offering a platform for the design of sophisticated materials. In structural applications, sequence-controlled polypeptides can be engineered to achieve specific mechanical strength and flexibility.[5,6] This also enhances their appeal for biomedical and sustainable applications.[7,8] From the perspective of functional materials, polypeptides are notable for their ability to be tailored to exhibit specific biological functions, such as drug delivery and tissue regeneration.[9−11]

In conventional peptide synthesis, methods such as ring-opening polymerization of amino acid *N*-carboxyanhydrides (NCAs),[12−14] solid-phase peptide synthesis (SPPS),[15−17] and liquid-phase peptide synthesis (LPPS)[18,19] have been widely employed. However, these methods have certain drawbacks. For example, protection and deprotection of functional groups are needed for amino acids with functional groups on their side chains. The process, including protection/deprotection and purification of the intermediates, requires substantial amounts of organic solvents, which have adverse environmental impacts. Both SPPS and LPPS offer the flexibility to synthesize polypeptides with any desired sequence; however, protection and deprotection steps are required, similar to NCA ring-opening polymerization. From the perspective of green chemistry, there is an increasing demand for the development of a polypeptide synthesis method that eliminates the need for side-chain protection, with attention to sustainability and environmental friendliness.

In response to the growing demand for environmentally sustainable methods for synthesizing polypeptides, we have directed our research toward environmentally conscious approaches, particularly focusing on chemoenzymatic polymerization (CEP).[2,20,21] CEP involves the utilization of proteolytic enzymes to catalyze the formation of polypeptides from amino acid esters, which represents a direct and environmentally friendly synthetic route in aqueous environments.[2,20−26] This method facilitates the facile synthesis of polypeptides with controlled sequences utilizing dipeptides or tripeptides as monomeric units.[27−31] In our investigations, we have successfully applied this strategy to incorporate amino acids with limited enzymatic recognition and nonnatural amino acids, such as 2-aminoisobutyric acid (Aib), aromatic monomers, and nylon monomers, into polypeptide backbones.[32−35] Capitalizing on the remarkable stereoselectivity and substrate specificity of enzymes, we have demonstrated the feasibility of polymerizing monomers bearing reactive side

Received: September 6, 2024
Revised: November 8, 2024
Accepted: November 18, 2024
Published: December 16, 2024

chains, such as serine,[36] arginine,[37] and tyrosine,[38] without applying side-chain protection strategies. Our CEP method provides an environmentally sustainable and versatile approach to polypeptide synthesis, with applications ranging from drug delivery to biomaterials, while simplifying the process by eliminating the need for side-chain protection.[39−41]

To perform efficient aminolysis reactions catalyzed by enzymes, prior activation of the C-terminus of amino acids or peptides, such as via esterification, is necessary. Various esters, such as methyl esters, ethyl esters, and benzyl esters, have been utilized as monomers in CEP. Since nonesterified carboxylic acids do not undergo polymerization, corresponding esters must be prepared to achieve the desired polypeptide via CEP.[24] Esterification of carboxylic acids via various organic chemical methods can be relatively straightforward.[42−46] For instance, acid- or base-catalyzed esterification is widely applied in fields like biodiesel production, where reducing environmental impact is essential.[47,48] Hence, we conceived the idea of conducting CEP in a one-pot manner following esterification of amino acids or oligopeptides, thereby eliminating isolation and purification steps. Direct synthesis of polypeptides from fully unprotected amino acids or oligopeptides not only reduces the environmental burden and labor associated with isolation and purification but also simplifies the acquisition of raw materials and facilitates the construction of libraries of diverse peptides prepared via CEP. As a preliminary step toward this one-pot CEP from unprotected amino acids, we attempted to synthesize poly-L-lysine (polyLys). PolyLys, which is a polypeptide bearing amino groups in its side chains, holds promise as an alternative to many widely used cationic polymers.[49] While ε-linked polyLys is typically biosynthesized in bacteria,[50,51] α-linked polyLys is chemically synthesized with the protection of side-chain amino groups.[52,53] In contrast, owing to the substrate specificity of enzymes, polyLys can be readily synthesized without side-chain protection. This aspect underscores its importance as a prime candidate for one-pot synthesis via CEP. In this study, we demonstrated the one-pot synthesis of polyLys by esterification of L-lysine (Lys-OH) followed by CEP of the resulting ester, providing facile access to α-linked polyLys.

MATERIALS AND METHODS

Materials

Papain (EC No. 3.4.22.2) was purchased from Sigma−Aldrich (St. Louis, MO, USA) and used as received. The activity was approximately 3 U/mg, where one unit hydrolyzes 1 μmol of *N*-benzoyl-L-arginine *p*-nitroanilide per minute at pH 6.2 and 25 °C. L-Lysine hydrochloride (Lys-OH·HCl) was purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan) and used without purification. Ethanol (99.9%) and dimethyl sulfoxide (DMSO, 99.0%) were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan) and used as received. For all the experiments, water was distilled and ion-exchanged prior to use. Deuterated water (D_2O) was purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). The other chemicals were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and used as received without purification unless otherwise noted.

Esterification of Lys with Ethanol

Lys-OH·HCl was dissolved in alcohol at various Lys concentrations. Then, an appropriate amount of 5 M HCl or sulfuric acid was added to the solution. The mixed solution was poured into a three-neck round-bottom flask with a magnetic stirrer and set to react under reflux conditions. The reaction proceeded for 24 h, after which the resulting solution was cooled to room temperature. The resulting solution was evaporated to remove the solvent. The resulting chemical compound in the dried state was used as a monomer for CEP, with papain used as a catalyst.

General Procedure of Chemoenzymatic Polymerization after Esterification of Lys with Ethanol

CEP was conducted after esterification of Lys-OH with alcohol. First, a phosphate buffer (1.0 M, pH 8.0) was added to the dry compound formed by esterifying Lys-OH with alcohol. At this time, the final concentration of Lys-OH and Lys derivatives was adjusted to 1.0 M. Then, the pH of the monomer solution was adjusted to various pH values (7.0, 7.5, 8.0, 8.5, and 9.0) by adding NaOH aq. (5.0 M). The monomer solution was poured into a glass tube equipped with a magnetic stirrer and set to react (ChemiStation, AYELA, Tokyo, Japan) at 40 °C under stirring at 800 rpm for 4 h. The water-soluble part in the resulting solution was purified via ultrafiltration using an Amicon Ultra (Merck, MWCO: 3k, 20,000*g*, 15 °C, 30 min) to remove the papain. The collected filtrate solution was then characterized via matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI−TOF MS), electrospray ionization mass spectrometry (ESI−MS) and reversed-phase high-performance liquid chromatography (RP−HPLC).

Analysis

Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{1}H NMR spectra were recorded on a Bruker DPX-400 spectrometer (Karlsruhe, Germany) at 25 °C and 400 MHz. The lyophilized polyLys was dissolved in D_2O and acidified with hydrochloric acid.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectroscopy (MALDI−TOF MS). MALDI−TOF mass spectrometric analysis was conducted using an ultrafleXtreme MALDI−TOF spectrophotometer (Bruker Daltonics, Billerica, MA) operating in linear positive ion mode. The sample was dissolved in trifluoroacetic acid (TFA) or water containing 0.1% TFA and mixed with a solution of α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile. The reaction solution, which formed no precipitate, was purified via ultrafiltration using an Amicon Ultra unit (Merck, MWCO: 3k, 9,000*g*, 25 °C, 45 min) to remove the papain. The collected filtrate solution was desalted using a Zip Tip (Merck, 0.6 μL, C_{18}) and then characterized via MALDI−TOF MS to identify the water-soluble product.

Reversed-Phase High-Performance Liquid Chromatography (RP−HPLC). RP−HPLC analysis was performed on an HPLC system consisting of an AS-2055 autosampler, a PU2089 gradient pump, a CO-4060 column oven, a UV-4075 UV/vis detector, and a PU-2089 Plus quaternary gradient pump (JASCO, Tokyo, Japan). The mobile phase was composed of acetonitrile (eluent A) and Milli-Q water containing 0.1 v/v% heptafluorobutyric acid (HFBA) (eluent B). The polyLys sample solution was injected and eluted with a mixed mobile phase with a linear gradient from A/B = 5/95 to A/B = 55/45 over 30 min at a flow rate of 0.5 mL min^{-1} at 25 °C. Each peak in the chromatograph was assigned, and the sum of the peak areas assignable to polyLys was compared with that of the monomeric peak to determine the monomer conversion.

https://doi.org/10.1021/acspolymersau.4c00073
ACS Polym. Au 2025, 5, 26−34

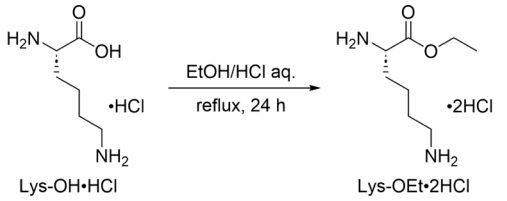
RESULTS AND DISCUSSION

Esterification of Lys-OH with Ethanol

To achieve high yields of high-molecular-weight polyLys, efficient activation of Lys is essential. Hence, we explored esterification using alcohol and acid catalysts. While an impressive 82% conversion was reported for a methyl esterification example using concentrated hydrochloric acid in 2,2-dimethoxypropane,[54] we opted for ethyl esterification using ethanol (EtOH) because of its cost-effectiveness and lower environmental impact as the preactivation step for CEP. Fischer esterification,[55] which is a well-known method for synthesizing esters from carboxylic acids and alcohols under acidic conditions, was selected. This reversible reaction allows ester formation by using excess alcohol and removing the generated water from the system. In this study, hydrochloric acid was used as the acid catalyst. Not only did hydrogen chloride serve as an acid catalyst, but also, water acted as a solvent conducive to the solubility of Lys-OH, which has limited solubility in EtOH.

Esterification of Lys-OH with EtOH was carried out at various EtOH volume fractions and Lys-OH concentrations (Scheme 1). The yield of L-lysine ethyl ester (Lys-OEt) was determined via ^{1}H NMR spectroscopy (Figure 1). The ^{1}H NMR spectrum of the resulting solution after the reaction with an EtOH volume fraction of 95% and an initial concentration of Lys-OH of 0.2 M is shown along with their peak assignments in Figure 1. In the ^{1}H NMR spectrum, the peaks of the α-protons of Lys-OEt (a) appeared at lower magnetic field than those of the α-protons of Lys-OH (a′). The yield was calculated from the integration ratio of the peaks of these α-protons (a, a′) and the methylene protons of the ethyl ester group (f). Since esterification catalyzed by acid is a reversible reaction, strategies such as water removal are commonly employed to shift the equilibrium toward ester formation and increase yield. In the present study, however, due to the solubility of Lys-OH, it was impossible to completely remove water. Therefore, to minimize the impact of the reversible reaction, we adjusted the solvent composition to reduce the water content (i.e., the concentration of hydrochloric acid) as much as possible during the esterification process. Interestingly, although excess EtOH favored the reversible esterification reaction, when the EtOH volume fraction exceeded a certain amount, the Lys-OEt yield decreased (Figure 2). This phenomenon may be attributed to the reduced solubility of Lys-OH in the reaction solution, causing Lys-OH to remain in the solid state after the esterification reaction. When the initial concentration of Lys-OH was 4.0 M, a 15% yield was achieved at an EtOH volume fraction of 40%. As the initial concentration of Lys-OH decreased to 3.0, 2.0, 1.0, and 0.5 M, the Lys-OEt yields reached their respective maximum values at EtOH volume

Scheme 1. Esterification of Lys-OEt with EtOH Catalyzed by HCl



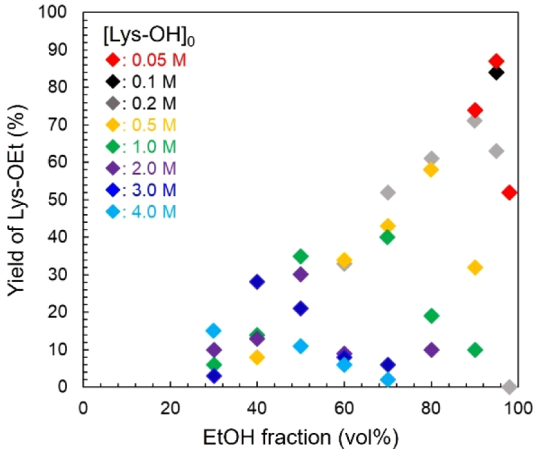


Figure 2. Effect of the EtOH volume fraction and Lys-OH concentration on the yield of Lys-OEt in the esterification of Lys-OH with EtOH catalyzed by HCl. The Lys-OEt yield was determined via ^{1}H NMR spectroscopy.

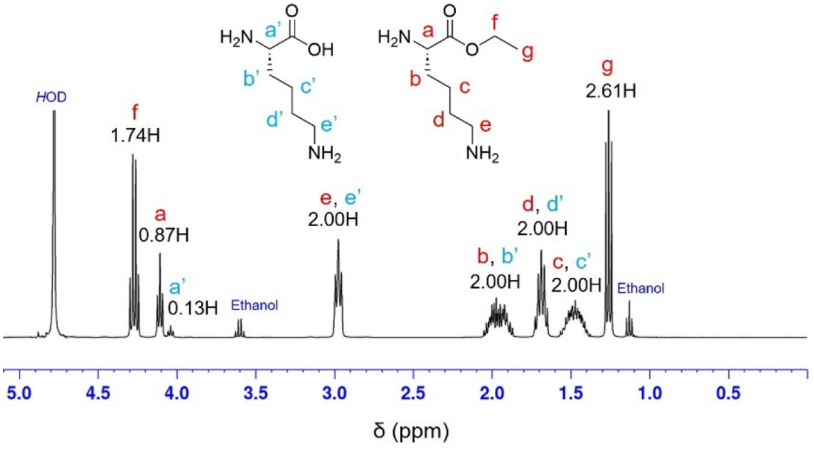


Figure 1. ^{1}H NMR spectrum of the resulting solution from the esterification of Lys-OH with EtOH catalyzed by HCl. D_2O was used as a solvent for ^{1}H NMR. The esterification conditions were $[Lys\text{-}OH]_0$ = 0.05 M and [EtOH/HCl aq.] = 95/5. The reaction was conducted under reflux of EtOH for 24 h.

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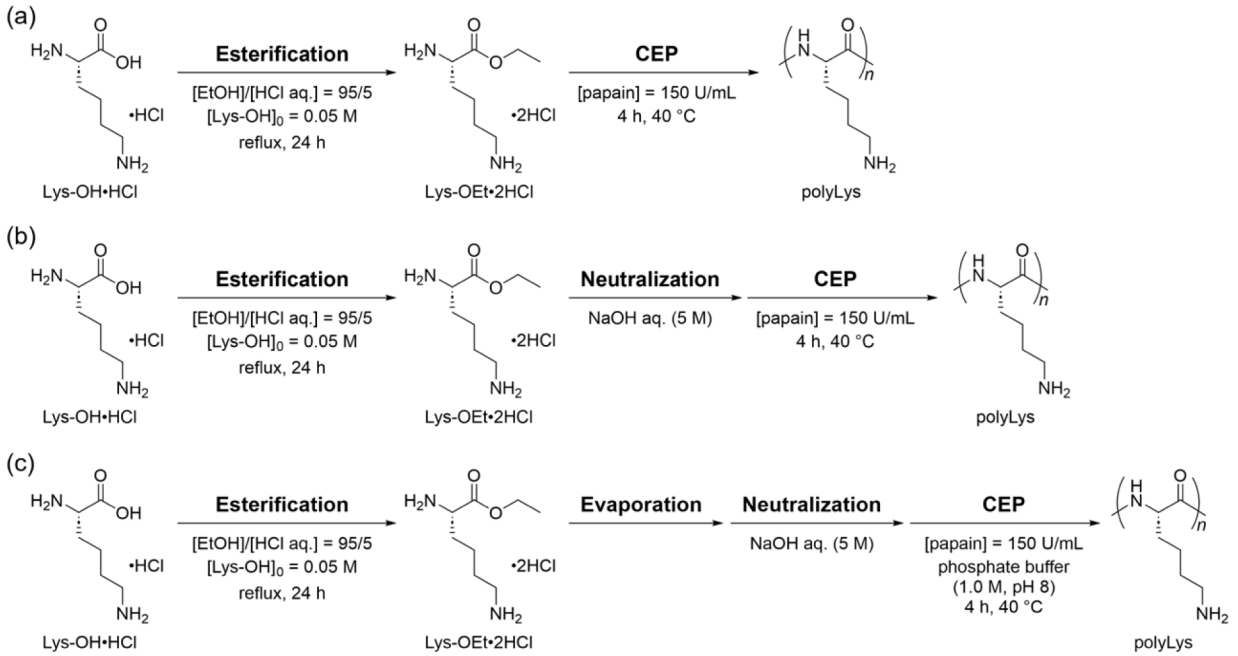
Scheme 2. One-Pot CEP of Lys-OEt Following Esterification of Lys-OH in EtOH



fractions of 50, 50, 70, 80, and 90%, with yields of 28, 30, 35, 58, and 74%, respectively, indicating an increase in the yield with increasing EtOH content. These observations highlight that esterification of Lys-OH at a low concentration (0.05 M) with a high EtOH volume fraction (95%) provided optimal conditions for obtaining high-purity Lys-OEt (87%).

CEP of the Resulting Lys-OEt without Isolation or Purification

One-pot CEP was initiated followed esterification of Lys with ethanol. Prior to CEP, the optimal conditions for esterification of Lys-OH with EtOH ($[\text{Lys-OH}]_0$ = 0.05 M, [EtOH]/[HCl aq.] = 95/5), as determined above, were applied. Papain was subsequently introduced into the resulting esterification solution to catalyze the CEP of Lys-OEt (Scheme 2a). Papain was selected for this study despite the demonstrated effectiveness of bromelain, α-chymotrypsin, and trypsin in catalyzing the CEP of Lys-OEt[56] because of its established ability to catalyze the polymerization of various amino acids,[29,36,57−59] which suggests potential prospects for future applications, such as copolymerization with these amino acids. However, owing to the highly acidic conditions of the solution after esterification, CEP failed to initiate under these conditions, necessitating neutralization of the esterification solution prior to CEP.

Papain-mediated CEP was conducted at 40 °C for 4 h by neutralizing the solution with 5 M NaOH aq. after esterification of Lys-OH, followed by carrying out the reaction at pH 8 (Scheme 2b). ESI−MS analysis of the resulting solution revealed the formation of oligoLys with degrees of polymerization (DPs) ranging from 2 to 4 through CEP (Figure S1). Polymerization did not occur in the absence of papain, suggesting that the identified oligoLys were generated through the reaction catalyzed by papain. To increase the DP, we systematically adjusted the reaction pH from 2 to 10. However, the obtained oligoLys exhibited consistent DPs throughout this pH range (Figure S1).

The use of methanol (MeOH), a protic solvent, has been reported to adversely affect the activity of papain.[60] Similarly, the residual EtOH used in the esterification of Lys-OH may decrease the enzymatic activity of papain during CEP in this study. In addition, when the resulting esterification solution is directly used for polymerization, elevating the monomer concentration to beyond that employed during esterification, which is a crucial parameter in CEP, is impractical. Generally, higher monomer concentrations confer a significant advantage by accelerating the reaction rate in polycondensation. Hence, prior to the addition of papain, the solvent was removed by evaporation after esterification (Scheme 2c). Subsequently, phosphate buffer (1.0 M, pH 8.0) was added to adjust the monomer concentration to 1.0 M, and the pH was adjusted to the desired value using a 5 M NaOH solution before initiating CEP by adding papain. The initial pH was varied from 7.0 to 9.0, and the DP of the resulting polyLys for each solution was determined via MALDI−TOF MS, whereas the monomer conversion to polyLys was calculated via RP−HPLC. MALDI−TOF mass spectra were obtained upon ultrafiltration to remove papain from the solution resulting from CEP, followed by crude purification using a C18 column. The maximum DPs from the MALDI−TOF mass spectra (Figure S2) and the conversions obtained from the RP−HPLC data (Figure S3) were plotted against the pH during CEP, as shown in Figure 3. The highest conversion was observed at pH 7.0, which gradually decreased with increasing pH and halved at pH 9. The hydrolysis of Lys-OEt would accelerate under higher pH conditions, diminishing the pool of monomers available for CEP and resulting in a decrease in the conversion to polyLys. Conversely, the maximum DP was the highest at pH 8.5 and decreased for both lower and higher pH values. In a detailed study of the effect of pH on the CEP of Ser-OEt, the highest polySer yield was observed at pH 8.5.[36] Considering this result in conjunction with the findings of this study, the optimal pH for papain-catalyzed polymerization was likely 8.5.

In the MALDI−TOF mass spectrum of the solution after CEP at pH 8.5, two series of peaks at intervals of 128 m/z were

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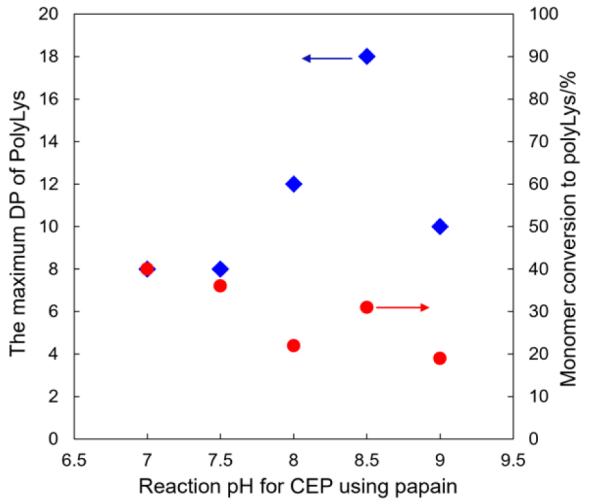

Figure 3. Maximum DP of the resulting polyLys and monomer conversion to polyLys in the CEP of Lys-OEt (1.0 M) via esterification of Lys-OH at 40 °C for 4 h at various pH values. The DPs of the resulting polyLys and monomer conversions were determined via MALDI−TOF MS and ^{1}H NMR, respectively.

observed, corresponding to the residue mass of lysine (Figure 4a). The series of larger peaks derived from polyLys with ethyl ester terminal groups was attributed to water adduct species ($[M + H + H_2O]^+$). The occurrence of water adduct species is not common; however, this phenomenon could be attributed to the highly hydrophilic nature of polyLys.[61−63] The other series originated from polyLys with carboxylic acid terminal groups resulting from hydrolysis. This result suggested that competitive hydrolysis reactions occurred during CEP of Lys-OEt mediated by papain. For both series, the peak top DP was 6, and the maximum DP was 18. In addition, peaks attributed to the dehydration products of polyLys were observed, probably due to the formation of diketopiperazine or lactam through cyclization at the C-terminus of polyLys. The CEP solution was also characterized via RP−HPLC. Each peak in the chromatogram was fractionated and collected for characterization, with assignments made based on ESI−MS. The monomer conversion of the CEP conducted at pH 8.5 was determined to be 31% by calculating the ratio of the peak area attributed to monomeric species (Lys-OH and Lys-OEt) to that attributed to oligomeric and polymeric species (polyLys) (Figure S4). By adjusting the pH and papain concentration, we synthesized polyLys with higher DPs and yields starting from Lys-OH. The ^{1}H NMR spectrum is shown in Figure 4b. Based on the spectra of additionally synthesized dimers and trimers,[64] as well as those of commercially available α-linked polyLys and ε-linked polyLys (Figure S5), peak assignments were made. The peaks of the methylene protons of the esters of Lys-OEt (f) and polyLys (F″) overlapped with that of the α protons of the repeating units in polyLys (A). The significant decrease in the integral ratio of the peaks of the ester methylene protons (f and F″), along with the substantial increase in the integral ratio of the peak of the α protons of Lys-OH (a′), originally present at only 13%, indicated not only the contribution of Lys-OEt to the polymerization but also an appreciable amount reverting to Lys-OH via hydrolysis. Furthermore, the regioselectivity of amide formation was investigated via ^{1}H NMR spectroscopy. In the case of ε-linkages, peaks corresponding to methylene protons adjacent to the amino group at the ε-position typically appear at approximately 3.2 ppm (Figure S5). However, almost no peaks were observed in this region, indicating that the amino group at the ε-position was not involved in the polymerization process and that the polymerization proceeded exclusively via α-linkages with practically 100% regioselectivity. This phenomenon can be attributed to the substrate specificity of the protease.

Effect of the Ester Structure on the Efficiency of One-Pot CEP

The structure of the amino acid ester used as a monomer has been previously established to affect the yield of polypeptides obtained through CEP.[21,26,36] To investigate the impact of the ester structure on one-pot CEP, considering both the esterification efficiency and yield, we examined the use of methanol and propanol—primary alcohols—in addition to ethanol for esterification and subsequent CEP (Scheme S1). Esterification was carried out under conditions similar to those when using ethanol ($[Lys\text{-}OH]_0$ = 0.05 M, [alcohol]/[HCl aq.] = 95/5, reflux for 24 h). After esterification, the alcohol was removed by evaporation, the monomer concentration was adjusted to 1.0 M with phosphate buffer, and the pH was adjusted to 8.5 with 5 M NaOH aq. before polymerization with papain. The esterification and CEP conversions, along with the DP of the resulting polyLys observed via MALDI−TOF MS, are summarized in Table 1.

The esterification conversion when using propanol (PrOH) was approximately 90%, which was comparable to that obtained with EtOH (runs 2 and 3). The low conversion of 50% obtained with MeOH was attributed to the partial evaporation of MeOH at high reaction temperatures. The conversion from the monomer to polyLys after CEP was the highest, at over 30%, for MeOH and EtOH (runs 1 and 2), although the DP was the highest when EtOH was used. CEP also proceeded with PrOH, but the DP was not as high (run 3). The use of highly hydrophobic and bulky esters, such as benzyl esters, can improve the yields of polypeptides. Propyl ester is more hydrophobic than ethyl esters. For polypeptides such as polyGly or polyAla, which precipitate after CEP, the increased hydrophobicity and bulkiness might increase the yield. However, for water-soluble polypeptides such as polyLys, this effect may be less significant. Furthermore, ^{1}H NMR measurements revealed that all the resultant polyLys consisted of 100% α-linkages, indicating that the substrate specificity of the enzyme was effective regardless of the ester type.

Acid Catalysis in Esterification Prior to CEP

The efficiency of amino acid esterification, which is the initial step in one-pot CEP, is a crucial factor influencing the outcome of the subsequent CEP process. If the C-terminus of the monomer is a carboxylic acid rather than an ester, then the subsequent polypeptide chain elongation induced by enzyme-catalyzed polymerization will be hindered, leading to a lower DP in the resulting polypeptide. Therefore, we employed sulfuric acid as the acid catalyst for esterification and performed one-pot CEP (Scheme S2). Sulfuric acid is commonly used as an acid catalyst in many dehydration reactions. In the ethyl esterification of Lys-OH, the reaction proceeded with a high conversion of 91%. As with the use of HCl as the acid catalyst, the reaction mixture was subsequently neutralized with NaOH and adjusted to pH 8.5, followed by CEP using papain. The conversion rate from monomers to oligomers significantly improved to 65% compared with that when HCl was used as the acid catalyst. Although the resultant polyLys exhibited 100% α-bonding, MALDI−TOF MS

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ACS Polym. Au 2025, 5, 26−34

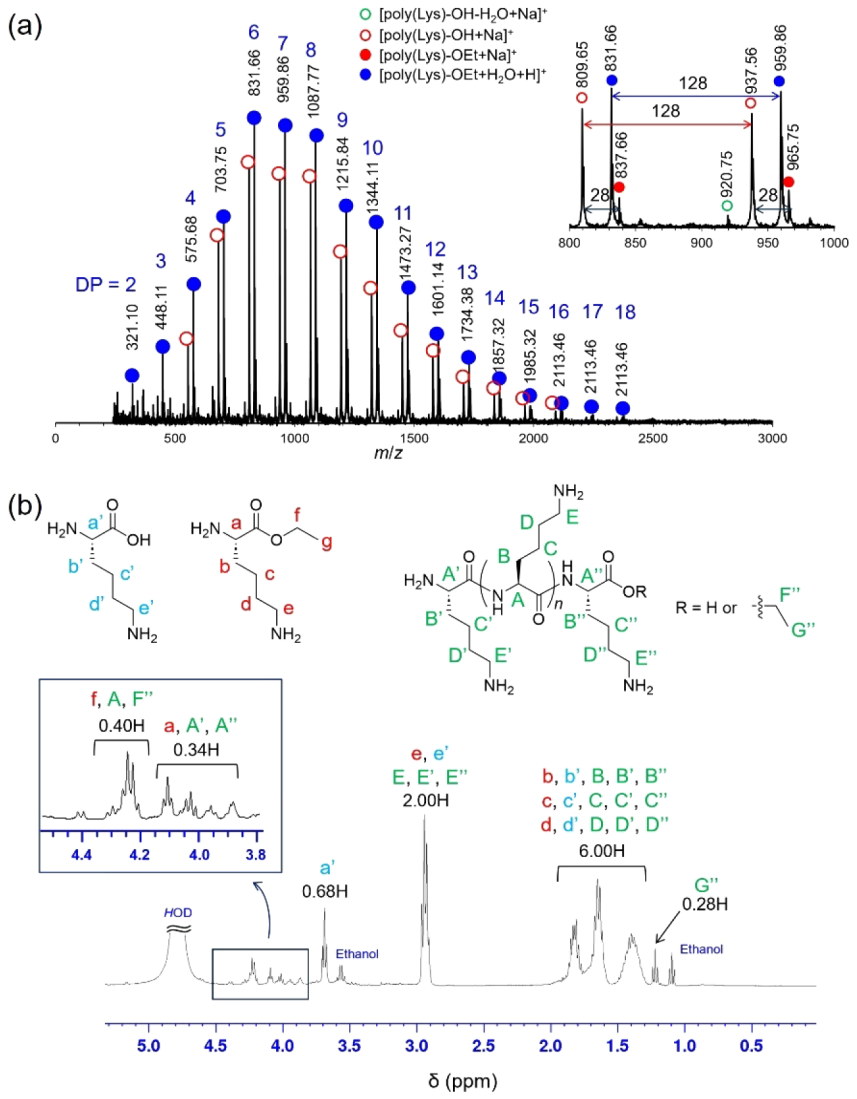

Figure 4. MALDI–TOF mass (a) and ^{1}H NMR (b) spectra of the solution resulting from CEP conducted at 40 °C for 4 h at pH 8.5. The sample was purified by ultrafiltration (MWCO: 3k) to remove the papain. D_2O was used as a solvent for ^{1}H NMR.

Table 1. One-Pot CEP of Lys-OH Using Various Alcohols[a]

	Esterification		CEP	
Run	Alcohol	Conv. (%)[b]	Conv. (%)[c]	DP[d]
1	MeOH	50	33	3–8
2	EtOH	87	31	3–18
3	PrOH	93	13	2–7

[a]Conditions: $[Lys\text{-}OH]_0$ = 0.05 M, [alcohol]/[HCl aq.] = 95/5, reflux for 24 h. [b]Determined by ^{1}H NMR. [c]Determined by RP–HPLC. [d]Determined by MALDI–TOF MS.

analysis revealed that the resulting polyLys had a peak top DP of 5 and a maximum DP of 10, indicating that its molecular weight was smaller than that obtained when HCl was used for esterification. While sulfuric acid is effective as an acid catalyst, it differs from HCl in that it does not volatilize with ethanol during evaporation and remains as an acid, potentially negatively affecting the enzyme reaction despite neutralization. Similarly, other commonly used acid catalysts, such as *p*-toluenesulfonic acid, were also likely to leave residual acidic traces that could interfere with the subsequent CEP step. For these reasons, gaseous HCl was considered the most suitable acid catalyst for the esterification step in the one-pot CEP, and therefore, additional acid catalysts were not investigated further. That said, given the need to volatilize or neutralize the acid in our system, employing insoluble neutralization agents, such as ion-exchange resins, might enhance the reaction system by addressing recycling and environmental concerns.

CONCLUSION

We demonstrated one-pot CEP for lysine by first esterifying the carboxyl groups with an acid catalyst and then performing polymerization with papain without isolating the ester. The highest esterification conversion was achieved when 5% hydrochloric acid was added to EtOH. Subsequent evaporation of the EtOH and adjustment of the pH to 8.5 with NaOH, followed by CEP resulted in polyLys with a peak top DP of 6 and a maximum DP of 18, with a conversion of 31%. We also conducted similar one-pot CEP experiments using different alcohols, including MeOH and PrOH. The ethyl ester-based one-pot CEP yielded the best results in terms of the DP and conversion. Given the moderate boiling point of EtOH and its ease of evaporation before CEP, the one-pot reaction utilizing EtOH appears to be the most efficient. In all the cases, the

https://doi.org/10.1021/acspolymersau.4c00073
ACS Polym. Au 2025, 5, 26–34

resulting polyLys was α-linked, demonstrating effective regioselective polymerization by papain. This synthetic method, which employs one-pot polymerization of amino acid esters without the need to isolate individual monomers, represents a significant advancement in the field of polypeptide synthesis. Based on several studies which highlight the regioselectivity and substrate specificity of papain in polymerizations of various amino acid esters, we believe this approach is generally applicable to substrates beyond lysine, as shown with corresponding ester derivatives. By eliminating the requirement for preisolated monomers, this approach not only simplifies the synthesis but also enhances its efficiency. It is particularly valuable for the polymerization of specialized amino acids or oligopeptides that are not readily commercially available as esters. Consequently, this method greatly expands the possibilities for creating a wide range of polypeptides with varied and complex structures. Such versatility would open new avenues for developing novel materials and biopolymers with tailored properties for application in fields ranging from materials science to biotechnology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acspolymersau.4c00073.

Figure S1: ESI−MS results of the resulting solution after CEP of Lys-OEt in one pot using papain; Figure S2: MALDI−TOF mass spectra of the solution resulting from CEP; Figure S3: representative RP−HPLC chromatogram and ESI−MS results of each HPLC fraction; Figure S4: HPLC chromatogram of polyLys obtained by esterification of Lys-OH in ethanol with 5% HCl aq.; Figure S5: ^{1}H NMR spectra of Lys-OEt, α-linked polyLys, and ε-linked polyLys, all of which were purchased; Scheme S1: one-pot CEP of Lys-OEt following esterification of Lys-OH with various alcohols catalyzed by HCl; Scheme S2: one-pot CEP of Lys-OEt following esterification of Lys-OH with EtOH catalyzed by sulfuric acid (PDF)

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K.N. contributed to conceptualization; K.T. and K.N. contributed to methodology; K.T. contributed to analysis; K.T. contributed to writing-original draft preparation; K.T. and K.N. contributed to writing-review and editing; K.T. contributed to visualization; K.T. and K.N. contributed to supervision; K.N. contributed to project administration; K.N. contributed to funding acquisition. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare the following competing financial interest(s): Alexandros Lamprou is an employee of BASF SE.

■ ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid from JST ERATO Grant No. JPMJER1602 for K.N., JST PRESTO Grant No. JPMJPR21N6 for Kousuke T., Grant-in-Aid for Transformative Research Areas (B) Grant No. JP20H05735 for K.N., and JSPS KAKENHI Grants No. JP20K05636 for Kousuke T. and No. JP23K04838 for Kayo T. This work was partly supported by the Asahi Glass Foundation for Kousuke T. The authors acknowledge the support of the BASF Network for Asian Open Research (NAO).

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