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Article

Chemoenzymatic Polymerization of L-Serine Ethyl Ester in Aqueous Media without Side-Group Protection

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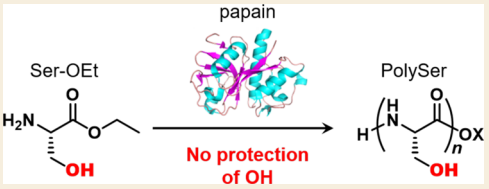
ABSTRACT: Poly(L-serine) (polySer) has tremendous potential as a polypeptide-based functional material due to the utility of the hydroxyl group on its side chain; however, tedious protection/deprotection of the hydroxyl groups is required for its synthesis. In this study, polySer was synthesized by the chemoenzymatic polymerization (CEP) of L-serine ethyl ester (Ser-OEt) or L-serine methyl ester (Ser-OMe) using papain as a catalyst in an aqueous medium. The CEP of Ser-OEt proceeded at basic pH ranging from 7.5 to 9.5 and resulted in the maximum precipitate yield of polySer at an optimized pH of 8.5. A series of peaks detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry revealed that the formed precipitate consisted of polySer with a degree of polymerization ranging from 5 to 22. Moreover, infrared spectroscopy, circular dichroism spectroscopy, and synchrotron wide-angle X-ray diffraction measurements indicated that the obtained polySer formed a β-sheet/strand structure. This is the first time the synthesis of polySer was realized by CEP in aqueous solution without protecting the hydroxyl group of the Ser monomer.



KEYWORDS: *polypeptide, poly(L-serine), chemoenzymatic polymerization, protecting group-free synthesis, β-sheet/strand*

INTRODUCTION

Polypeptides, a linear chain of amino acids linked by peptide bonds, are potentially applicable in the biological, pharmaceutical, and bioinspired material fields.[1−4] Polypeptides can fold into functional higher-order structures depending on various amino acid sequences.[5−7] Such structural diversity provides fascinating properties, including a high affinity to genes and proteins, highly selective/efficient intracellular uptake, high mechanical strength inspired by natural materials, and distinctive assembled structures.[8−10] Among polypeptides, poly(L-serine) (polySer) is an attractive hydrophilic polypeptide for various applications. Since L-serine (Ser) has a hydroxyl group in the side chain, resulting in high hydrogen bonding ability, it plays a key role in designing the secondary structure of polypeptides.[11,12] Moreover, the reactive hydroxyl group of Ser can be used as a reaction site for the posttranslational modification of polypeptides or proteins.[13−17] For instance, antibody-free detection systems for cancer have been demonstrated by utilizing the phosphorylated reaction of the hydroxyl groups of Ser in polypeptides.[17] The hydroxyl group of Ser can allow the construction of bioconjugates with phenylboronic acids, leading to a smart sensor for proteins.[14] Therefore, due to its high applicability in various fields, the facile and convenient synthesis of polySer on a large scale has been desired.

Various polypeptide synthesis techniques, including liquid-phase synthesis,[18,19] solid-phase synthesis,[20,21] and ring-opening polymerization,[22,23] have been developed. However, to the best of our knowledge, there is no report to date on the synthesis of polySer from Ser monomers without a protecting group on the hydroxyl group. Ring-opening polymerization of the α-amino acid *N*-carboxy anhydrides (NCAs) of Ser provides high molecular weight polySer with good control of the molecular weight and polydispersity.[11,24−32] However, NCA syntheses require toxic agents such as triphosgene; additionally, a condensing agent is required for appropriate monomer synthesis. Liquid-phase and solid-phase peptide syntheses as well as NCA ring-opening polymerization require a large amount of organic solvent for synthesis, purification, and protection/deprotection. In all existing methods, the hydroxyl group of the Ser monomer should be protected in advance to prevent undesired reactions on the hydroxyl group; thus, various *O*-protected Ser monomers, such as *O*-benzyl,[25−27,31] *O*-pentenyl,[11] *O*-*tert*-butyl,[28−30,32] and *O*-acetyl[27] Ser derivatives, have been used for the synthesis of polySer. Although these synthesis methods are useful, the synthesis of polySer from a Ser monomer without side chain protection is desirable for practical material production in the context of atom economy.

In pursuit of the environmentally benign synthesis of polypeptides, we have focused on chemoenzymatic polymer-

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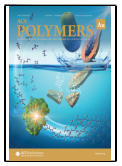

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ization (CEP) using a protease as a catalyst for the synthesis of various types of polypeptides, including star-shaped, deblocked, and structural protein-like polypeptides.[33−41] Unlike conventional synthesis methods, CEP proceeds in aqueous solution. Polymerization proceeds in a highly regio- and stereoselective manner due to the substrate specificity of enzymes.[42,43] Therefore, polypeptides can be synthesized without any side chain protection of amino acid monomers. In addition, the enzyme is easily removed just by washing the resultant polypeptides with water, and the polymerization technique is scalable. The immobilized enzymes would also be useful for water-soluble polypeptides in terms of enzyme removal. Recently, we and other groups have demonstrated that the CEP of amino acid monomers with functional side groups can result in various polypeptides with reactive side groups, including poly(L-lysine), poly(L-cysteine), and poly(L-tyrosine), without the use of protecting groups.[44−50] Most importantly, in all the CEPs of these amino acid monomers, polypeptides consisting of α-peptide bonds were formed while maintaining the intact reactive side groups due to the regio- and stereoselectivity of protease-mediated reactions.[42]

In this study, the CEP of L-serine ethyl ester (Ser-OEt) was performed in aqueous media using papain as the enzyme catalyst. After the optimization of the reaction conditions, Ser-OEt without any protection on the hydroxyl group was directly converted to polySer via enzyme-catalyzed polymerization. Characterization of the chemical structure of the resultant product, including proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, revealed that polySer with a degree of polymerization (DP) ranging from 5 to 22 was obtained. The secondary structure of polySer was characterized by circular dichroism (CD), infrared spectroscopy (IR), and synchrotron wide-angle X-ray diffraction (WAXD) measurements. The results of this study should lead to the development of noteworthy research on the application of hydrophilic polypeptides.

MATERIALS AND METHODS

Materials

Papain was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received (EC Number: 3.4.22.2). The activity was approximately 3 U/mg, where one unit was defined as the amount of enzyme that hydrolyzed 1 μmol *N*-benzoyl-L-arginine ethyl ester per minute at pH 6.2 and 25 °C. Ser-OEt (99%) was purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). L-Serine methyl ester (Ser-OMe, 98.0%) was purchased from Tokyo Chemical Industry Corporation Ltd. (Tokyo, Japan). Trifluoroacetic acid (TFA, 98.0%), 2,2,2-trifluoroethanol (TFE, 99.0%), dimethyl sulfoxide (DMSO, 99.0%), *N*,*N*-dimethylformamide (DMF, 99.5%), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 99.5%) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and used as received. For all experiments, water was distilled and ion-exchanged prior to use.

Synthesis of polySer

The CEP of Ser-OEt or Ser-OMe was performed according to the following typical procedure. Initially, Ser-OEt hydrochloride (2 mmol, 0.3392 g) or Ser-OMe hydrochloride (2 mmol, 0.3112 g) was dissolved in phosphate buffer (1 M, pH 8.0, 2 mL). Then, the pH of the monomer solution was adjusted to an appropriate pH (6.5−10.0) using 5 M hydrochloric acid (HCl) or 5 M sodium hydroxide (NaOH) aqueous solution. The monomer solution was poured into a glass tube equipped with a magnetic stirrer, set in a reaction device (ChemiStation, AYELA, Tokyo, Japan) at 40 °C and stirred at 800 rpm. A powder of papain (0.100 g, 50 mg/mL) was then added to the monomer solution to start polymerization. The solution was stirred at 40 °C and 800 rpm for 4 h. The resultant solution was centrifuged (20,000 ×*g*, 15 °C, 1 min) to separate the formed precipitate from the crude solution. The collected precipitate was washed for at least three cycles of centrifugation/redispersion using deionized water. The resulting pellet was then lyophilized to yield polySer. The chemical structure of the product was characterized by ^{1}H NMR spectroscopy and MALDI-TOF MS spectrometry.

The supernatant of the reaction solution after collecting the precipitate was purified with ultrafiltration using an Amicon Ultra unit (Merck, MWCO: 3 k, 20,000 × *g*, 15 °C, 30 min) to remove the papain. The collected filtrate solution was lyophilized and then characterized by electrospray ionization (ESI) mass spectrometry to identify the water-soluble product.

TFA Treatment of polySer

To characterize the secondary structure of polySer in an aqueous solution, the precipitated polySer, which is insoluble in water, was solubilized in aqueous medium by a subsequent TFA treatment. The lyophilized precipitate obtained by the CEP of Ser-OEt was dissolved in excess amounts of TFA. After dissolving, an excess amount of diethyl ether was added to reprecipitate from the TFA solution. After removing the supernatant solution, the precipitates were washed three times by centrifugation/redispersion using fresh diethyl ether. The remaining precipitates were dried under reduced pressure for 1 h to obtain a white solid. The polySer solid after TFA treatment was easily dissolved in aqueous media and used for further characterization by CD spectroscopy.

Characterization Procedure

^{1}H and ^{1}H-^{1}H COSY NMR spectra were recorded on a Bruker DPX400 spectrometer (Bruker, Bremen, Germany) at 400 MHz. Deuterated TFA (TFA-*d*) was used as the solvent. MALDI-TOF mass spectra were recorded with an AutoFlex III Plus (Bruker) spectrometer using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix dissolved in acetonitrile. The Fourier transform infrared (FT-IR) spectra of the collected precipitate samples were recorded by using an IR Prestige-21 Fourier transform infrared spectrophotometer (Shimadzu Corporation, Kyoto, Japan) with a MIRacle A single-reflection attenuated total reflection unit using a Ge prism. The sample for the FT-IR spectra was used before TFA treatment. ESI mass spectrometry was performed with the samples using an Exactive Plus Orbitrap ESI mass spectrometer (Thermo Fisher Scientific, MA, USA).

CD spectroscopic analysis was conducted using a JASCO J-820 CD spectropolarimeter (JASCO, Tokyo, Japan). Measurements were performed on polySer solutions (0.5 mg/mL) in deionized water using a cuvette with a 1 mm path at 25 °C. The pH of the sample solution was adjusted using 5 M NaOH or 5 M HCl. Each spectrum represents the average values of three independent scans from 195 to 290 nm with a resolution of 1 nm.

The WAXD measurement was performed at the BL05XU beamline (SPring-8, Harima, Japan) using an X-ray energy of 12.4 keV (wavelength: 0.1 nm). The obtained two-dimensional (2D) diffraction patterns were converted to one-dimensional (1D) profiles by azimuthal integration using Fit2D.[51] The synthesized materials were identified by ESI mass spectrometry (Thermo Fisher Scientific Exactive Plus Orbitrap ESI mass spectrometer, MA, USA).

RESULTS AND DISCUSSION

CEP of Ser-OEt Using Papain as the Catalyst

Proteases generally catalyze not only the hydrolysis of polypeptides but also the aminolysis of amino acid derivatives to afford polypeptides under certain reaction conditions, such as high monomer concentrations.[35] Appropriate acyl donors, such as amino acid esters, can kinetically promote the formation of a tetrahedral intermediate followed by nucleophilic attack by the amino group of another amino acid ester.[42]

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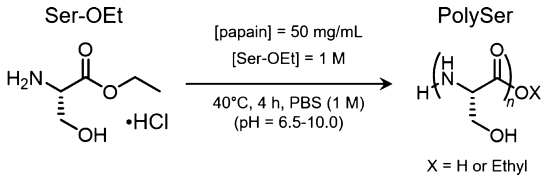
Since CEP utilizes the native function of the protease in the aminolysis of amino acid derivatives, the reaction pH also affects the aminolysis reaction efficiency. Therefore, in this study, we conducted the CEP of Ser-OEt using papain as a catalyst in an aqueous solution and investigated the reaction efficiency at various pH values (Scheme 1). Papain was selected as the enzyme because of its broad substrate specificity, including neutral amino acids.

Scheme 1. Papain-Catalyzed Polymerization of Ser-OEt



The CEP of Ser-OEt was carried out at various initial pH values between 6.5 and 10.0 at a monomer concentration of 1 M (Figure 1a) and resulted in the formation of a white precipitate from pH 7.5 to 9.5. This polymerization system should be a kind of a precipitation polymerization, since the monomer was soluble and the polymerized product became a precipitate in the polymerization medium. In contrast, no precipitate is observed during the synthesis of polySer by the techniques such as liquid-phase synthesis, solid-phase synthesis, and ring-opening polymerization, because the deprotection of the hydroxyl group of the polymers of *O*-protected Ser are performed in strong acids such as TFA or HBr.[25,27,29,30,32] In the CEP conducted at an initial pH below 7.0 or above 10.0, precipitation was hardly obtained after polymerization due to the formation of water-soluble oligo(L-serine) (oligoSer) (described in detail later). The precipitate was collected by centrifugation and washed with water to remove papain for a monomer concentration of 1 M. Judging from almost no peak other than that originating from polySer in ^{1}H NMR, we considered that papain was almost removed from the precipitate of polySer. Although a very small amount of papain or degraded residues of papain might remain in the precipitate, their little contamination would not affect the characterization of polySer. On the other hand, the CEP of Ser-OEt using 12.5 mg/mL of papain afforded no precipitate, suggesting the need of a papain concentration of 50 mg/mL even if the removal of papain would be more troublesome than at the lower concentration. The average yield of the precipitate at various pH values was determined (Figure 1b). When the CEP of Ser-OEt was conducted at slightly basic pH, the yield of the precipitate increased and reached a maximum of 20.4% at pH 8.5. This result revealed that the optimum pH to obtain the precipitate was pH 8.5. A time course study of the CEP of Ser-OEt was also performed at pH 8.5 and an initial Ser-OEt concentration of 1 M (Figure 2a). The precipitate rapidly appeared at approximately 30 min, and the yield of the precipitate increased to 20% within 60 min, exhibiting a similar tendency to the CEP of other amino acid derivatives.[36] After 60 min, the yield of the precipitate reached a maximum value and almost remained constant until at least 360 min, indicating that the hydrolysis of the polySer redissolved in the polymerization medium hardly occurred with increasing reaction time. This indicates that the redissolution of polySer might not occur once the precipitate is formed. In addition, the number average DP of the precipitated polySer obtained by the CEP of Ser-OEt after 60 min was calculated to be 7.1 by the integral ratio of peak a′ to a and a″ in the ^{1}H NMR spectrum (Figure S1a). This value was similar to that for the polySer obtained by the CEP of Ser-OEt after 360 min. The ESI MS spectrum of the water-soluble part in supernatant solution showed the peaks of small amounts of Ser-OEt as well as those of hydrated oligoSer (Figure S1b). These results indicated that the molecular weight of polySer got to the maximum within 60 min. In contrast, the polymerization at pH 7.0 provided no precipitation. The resultant solution was then adjusted to pH 8.5; however, no precipitation was formed (Figure S2). This result indicated that the precipitation of polySer after CEP was not caused by a change in the solubility of the resulting product and probably due to the low molecular weight of the resultant product. The pH dependence of the papain-catalyzed hydrolysis of *N*-acetyl-L-phenylalanylglycine *p*-nitroanilide was reported in the literature.[52] This result indicates that the polymerization activity of papain varies with pH, even though the optimal pH of aminolysis differs from the hydrolysis. The

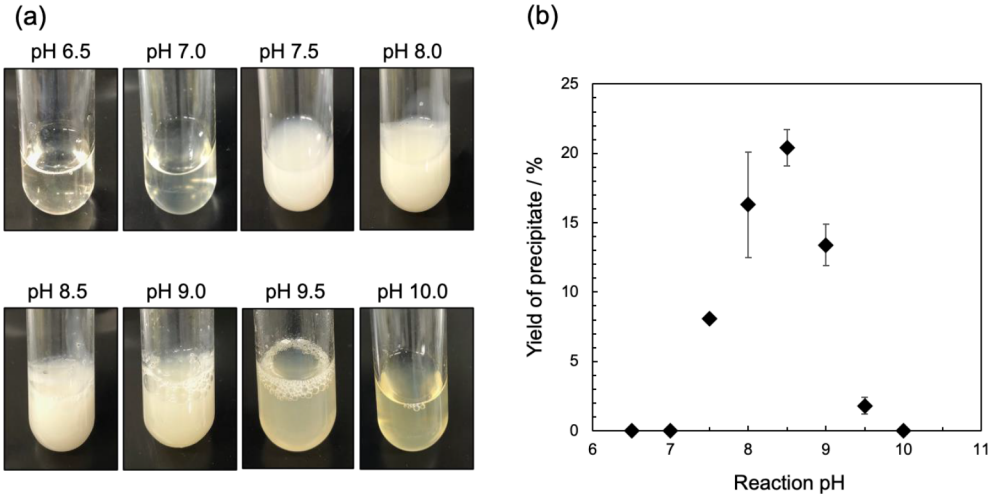


Figure 1. (a) Photographs of the resultant solution after the CEP of Ser-OEt at various pH conditions. (b) Yield of the precipitate formed after the CEP of Ser-OEt at various pH conditions. The precipitate was obtained as an insoluble part after washing with deionized water by the centrifugation/redispersion process to remove the remaining monomer and papain.

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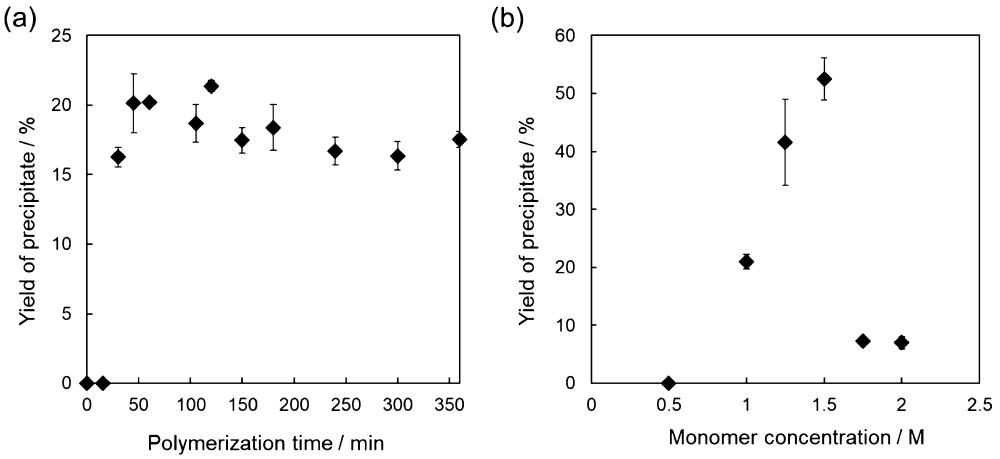

Figure 2. (a) Time course of the yield of the precipitate during the CEP of Ser-OEt conducted with papain (50 mg/mL) at pH 8.5. The monomer concentration was fixed at 1 M. (b) Yield of the precipitate for CEPs conducted at various monomer concentrations. The yield was an average of three independent experiments to determine the standard deviation (N = 3).

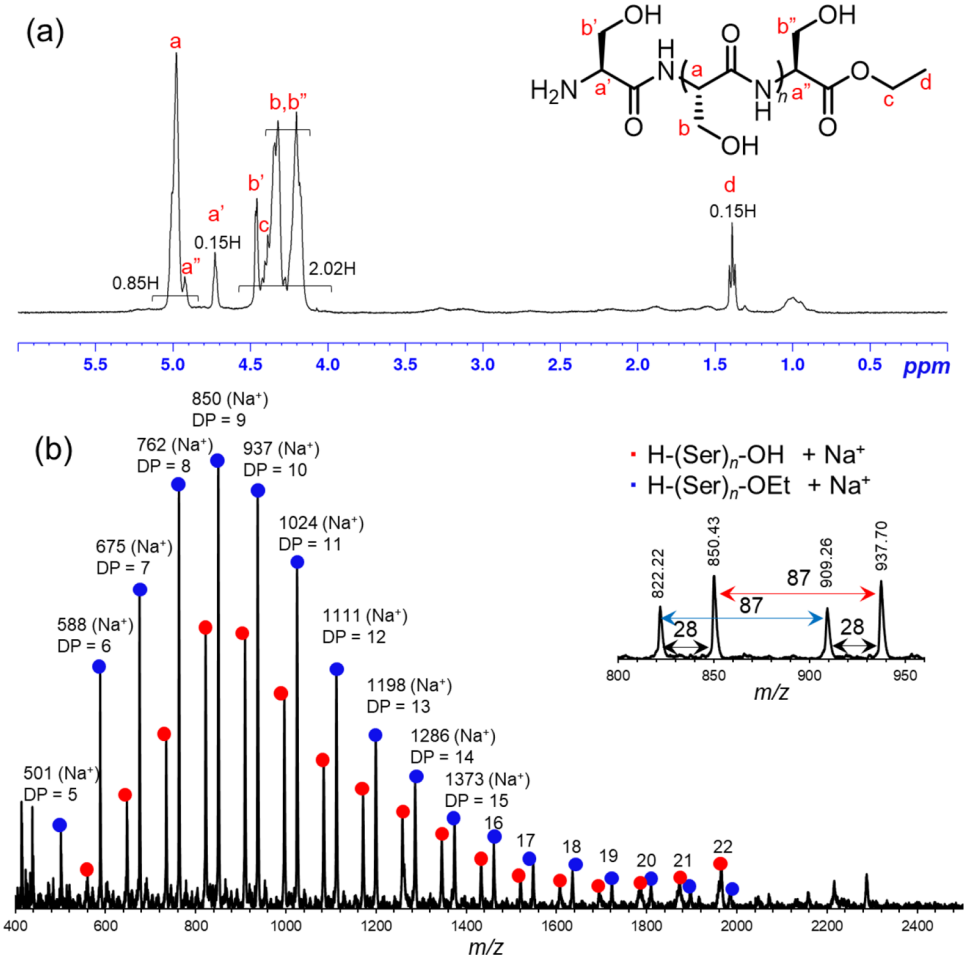

Figure 3. (a) ^{1}H NMR and (b) MALDI-TOF MS spectra of the precipitated polySer obtained by the CEP of Ser-OEt at pH 8.5. TFA-*d* was used as the solvent for ^{1}H NMR.

difference in the yield of polySer as a precipitate was determined by the difference in papain activity in addition to the reactivity of Ser-OEt at each pH, which is the case for typical CEPs.[35]

The initial concentration of monomer ($[M]_0$) affects the yield of polypeptides in CEPs.[34,39,42] Similarly, the $[M]_0$ of Ser-OEt significantly influenced the yield of the precipitate, as shown in Figure 2b. By optimizing the $[M]_0$ in a range of 0.5−2.0 M, the yield of the precipitate was improved from 20.4 to 56.9%. The maximum yield was obtained for the CEP conducted at $[M]_0$ = 1.5 M. It should be noted that the precipitate hardly formed for $[M]_0$ below 0.5 M or above 1.75 M, probably due to the formation of water-soluble oligoSer instead of polySer, even though the reaction pH was fixed at

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8.5. At 0.5 M Ser-OEt, papain might exhibit the lower substrate recognition against Ser-OEt, resulting in the formation of water-soluble oligoSer. At over 1.75 M of Ser-OEt, the papain concentration was not sufficient for the excess amount of Ser-OEt. Also, the larger decrease in the reaction pH was caused by the polymerization of excess amounts of Ser-OEt, resulting in the synthesis of oligoSer instead of polySer.

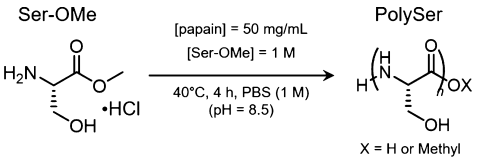
The collected precipitate was characterized by ^{1}H NMR and MALDI-TOF MS spectra (Figure 3). The chemical structure of the precipitate was characterized by ^{1}H NMR, and the protons of polySer were detected and assigned (Figure 3a). We assigned three peaks that appeared at 4.6−5.0 to the α-proton of polySer because they correlated to the peaks assigned to the methylene protons (indicated as b, b′, and b″ in Figure 3a), as determined by ^{1}H-^{1}H COSY NMR (Figure S3). The split peaks include the α-proton of the Ser residue at the N-terminus and C-terminus in addition to that of repeating units, as with the other polypeptides.[34] Moreover, the MALDI-TOF MS spectrum of the obtained precipitate for CEP at pH 8.5 revealed a series of peaks with an m/z interval of 87 corresponding to the Ser monomer unit (Figure 3b). Each peak (marked with blue dots in Figure 3b) matched the exact mass of polySer with an ethyl ester C-terminus. The most intense peak appeared at 850 m/z and was assigned to polySer with a DP of 9. The DP assignable to the series of peaks of polySer ranged from 5 to 22. In addition, the second largest series of peaks (marked with red dots in Figure 3b) had m/z values that were 28 less than the peaks of polySer with an ethyl ester C-terminus. These peaks were assigned to polySer with a hydrolyzed C-terminus. This result indicated that a competitive hydrolysis reaction occurred during CEP when using papain. The MALDI-TOF MS spectra for polySer obtained from pH 7.5 to 9.0 showed similar profiles, indicating that the DP of the precipitated polySer was hardly affected by the reaction pH during CEP (Figure S4). The water-soluble part of the supernatant after CEP was also characterized by ESI mass spectrometry. Notably, the formation of oligoSer with DP values from 2 to 4 was confirmed for the solution resulting from the CEP of Ser-OEt conducted at pH 7.0, 8.5, and 10.0 (Figure S5). Even though no precipitation was obtained from CEP conducted at pH 7.0 and 10 (Figure 1a), oligoSer formed as the water-soluble part after CEP (Figure S5a,c). The formation of oligoSer was also confirmed in the supernatant solution after the CEP of Ser-OEt at the optimal pH (pH 8.5) (Figure S5b). Collectively, the polymerization activity of papain was enhanced at the optimized pH, resulting in polySer as a precipitate. The polymerization probably competed with the hydrolysis reaction at the C-terminus of polySer at lower or higher pH to give only oligoSer, although the aminolysis reaction also occurred.

The molecular weight of the polySer obtained by the CEP of Ser-OEt was relatively low compared to that of the polySer obtained by the NCA ring-opening polymerization via the protection/deprotection process[29−32] probably due to the precipitation during the CEP. The addition of the solvent such as fluorinated solvents might be effective on the further increment of the molecular weight by avoiding the formation of the β-structure to prevent the precipitate of polySer. It was reported that the poly(L-phenylalanine) (polyPhe) with a high molecular weight (ca. 30,000) was obtained by enzymatic synthesis in 1,1,1,2-tetrafluoroethane using subtilisin Carlsberg as an enzyme.[53]

Reactivity of the Other Ester Monomer on CEP

The CEP of Ser-OMe was also conducted to compare the reactivity of the ester groups of the Ser monomer (Scheme 2).

Scheme 2. Papain-Catalyzed Polymerization of Ser-OMe



The precipitate gradually appeared after papain was added to the solution of Ser-OMe similar to the CEP of Ser-OEt, and the yield of the precipitate reached 29.0% after 240 min. The structure of the precipitate was determined by ^{1}H NMR and MALDI-TOF to confirm the synthesis of polySer from Ser-OMe (Figure 4). In the ^{1}H NMR spectrum, two peaks originating from the α-protons of polySer appeared at 4.6−5.0 ppm (indicated as a, a′, and a″ in Figure 4a) and were assigned by the cross peaks with the methylene protons (indicated as b, b′, and b″ in Figure 4a) in the ^{1}H-^{1}H COSY NMR spectrum (Figure S6). For polySer derived from Ser-OMe, the α-proton of the Ser residue at the N-terminus (indicated as a′ in Figure 4a) was divided from those of the repeating units and the C-terminus (indicated as a and a″ in Figure 4a). In addition, some hydrolysis of the methyl ester at the C-terminus occurred, judging from the integral values of the α-proton of the Ser residue at the N-terminus and the methyl proton of the methyl ester at the C-terminus. In the MALDI-TOF MS spectrum of the precipitate, there were two main repeating series of mass peaks with 87 m/z intervals corresponding to a Ser monomer unit (Figure 4b). The larger series of peaks was assigned to hydrolyzed polySer at the C-terminus (marked with red dots in Figure 4b), and the other was assigned to polySer with a methyl ester at the C-terminus (marked with blue dots in Figure 4b). The DP assignable to the series of peaks of polySer ranged from 7 to 24, and the highest peak was observed at 737 m/z, DP = 8.

The effect of the ester group of the monomers on the CEP was investigated. In the CEP of L-alanine (Ala) and glycine (Gly) esters, the ethyl esters exhibited higher yields than the methyl esters, while the DPs of the resultant poly(L-alanine) (polyAla) or poly(glycine) (polyGly) were comparable for the two ester monomers.[39] However, the CEP of Ser-OEt performed under the same condition ($[M]_0$ = 1.0 M, at pH 8.5, for 4 h) afforded a slightly lower yield (20.4%) than that of the CEP of Ser-OMe. For the DP of the resultant polySer determined by MALDI-TOF MS, the two were comparable. On the other hand, the number average degree of polymerization (DP_n) of polySer was determined by the integral ratio of the peak a′ against the peaks a and a″ in ^{1}H NMR spectra (Figures 3 and 4). The DP_n of polySer obtained by the CEP of Ser-OEt was calculated to be 6.6, while that obtained from Ser-OMe was calculated to be 14. For Ala and Gly, the affinity of their ethyl esters to papain might be higher than the methyl esters, resulting in a higher yield for the ethyl esters.[39] In contrast, the affinity of Ser-OMe to papain might be higher than Ser-OEt, resulting in a higher yield as well as a higher DP_n for the methyl ester. Ser is a hydrophilic amino acid having a hydroxyl group, whereas Ala and Gly are hydrophobic amino acid. Therefore, the trend of the affinity could be reversed. Moreover, considering that the main series in the MALDI-

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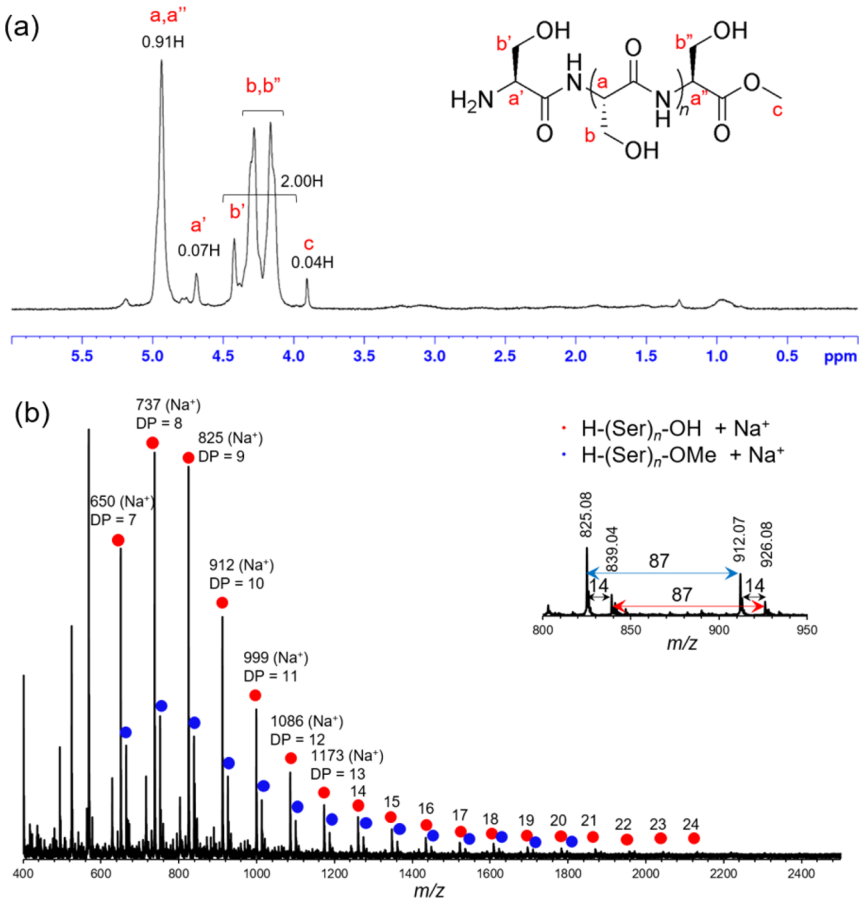

Figure 4. (a) ^{1}H NMR and (b) MALDI-TOF MS spectra of the precipitated polySer obtained by the CEP of Ser-OMe at pH 8.5. TFA-*d* was used as the solvent for ^{1}H NMR.

TOF MS spectrum of the polySer derived from Ser-OEt was assigned to the nonhydrolyzed polySer at the C-terminus in contrast to the polySer derived from Ser-OMe, the methyl ester might have been more easily hydrolyzed than the ethyl ester. A similar tendency was exhibited in the CEP of methyl esters and ethyl esters of Ala or Gly.

Solubility Test of polySer

Solubility testing was performed to determine a good solvent for the obtained polySer. The isolated polySer (10 mg, white powder) was dissolved in various solvents (1 mL) (Figure 5),

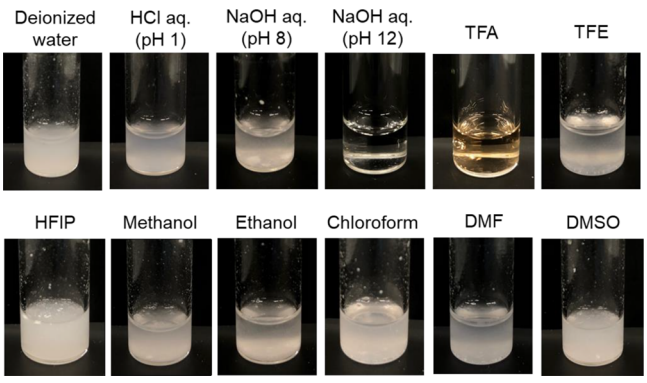

Figure 5. Solubility testing of the precipitated polySer (10 mg/mL). Photographs of the resultant solution after dissolving the polySer that formed during polymerization.

and the solubility was determined by its appearance. The obtained polySer was insoluble in common solvents such as alcohols and chloroform. Highly polar solvents such as DMSO and DMF and fluorinated solvents such as TFE and HFIP, which are often used as good solvents for polypeptides, were unable to completely dissolve polySer. A highly basic aqueous medium (pH 12.0) was a suitable solvent for the obtained polySer, whereas polySer did not dissolve in aqueous solution at pH 1.0 or 8.0. TFA was also a good solvent to completely dissolve the obtained polySer. Solubility testing at the lower concentration (1 mg of polySer was dissolved in 1 mL of various solvents) exhibited a similar tendency (Figure S7). On the other hand, the polySer obtained by the NCA ring-opening polymerization of *O*-protected Ser followed by the deprotection process was reported to be partly soluble in water, although the molecular weight of the polySer was estimated as 650 comparable for that obtained in this study.[29]

The obtained polySer was insoluble over a broad range of pH values from 1.0 to 8.0 but was soluble in a highly basic aqueous solution (pH 12). The pKa of the hydroxyl group of the Ser residue incorporated in the oligopeptide was 12.8, which was lower than that of the hydroxyl group in the Ser monomer (15.9).[54] Therefore, the hydroxyl groups in the side chain of polySer were deprotonated to some extent at pH 12, allowing polySer to dissolve in water despite tending to adopt a β-sheet structure (described in detail later). The obtained polySer was soluble in TFA and basic aqueous solution, promising further functionalization of polySer by post-modification of the free hydroxyl group on the side chain.

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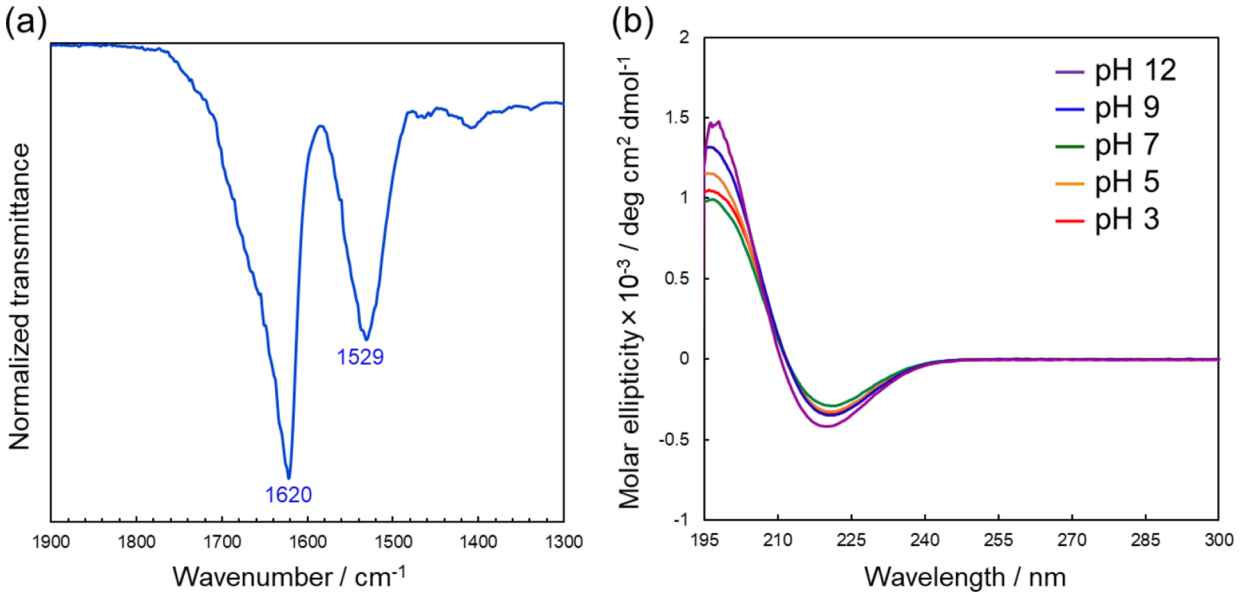

Figure 6. (a) IR spectrum of the precipitated polySer obtained after CEP at pH 8.5. (b) CD spectra of polySer in water at various pH values and 20 °C. The peptide concentration was 0.5 mg/mL.

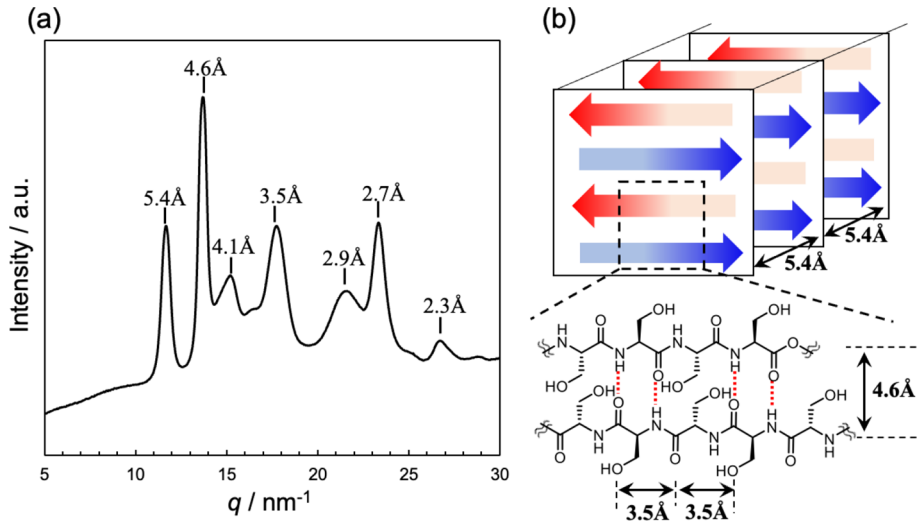

Figure 7. (a) 1D powder WAXD profile of polySer and (b) schematic illustration of the antiparallel β-sheet structure of polySer.

As shown in Figures 1a and 5, the synthesized polySer did not dissolve in aqueous solution except under highly basic conditions. However, once polySer was dissolved in TFA and then reprecipitated with diethyl ether, and the obtained solid was temporarily soluble in water at various pH values (Figure S8). Note that the dissolved polySer gradually precipitated from water again after a long time period, such as 1 week at 25 °C. The small amount of remaining TFA in polySer might influence the secondary structure or the hydrated state of polySer in aqueous solution, similar to the case of proteins.[55]

Characterization of the Secondary Structure of the Obtained polySer

To date, there are no reports of experimental characterization of the secondary structures of polySer at various pH values. To reveal the secondary structure of polySer, the precipitated polySer obtained by CEP was characterized by IR spectroscopy. The IR spectrum of the precipitated polySer from CEP conducted at pH 8.5 is shown in Figure 6a. The strong peak in the amide I region (1600−1700 cm^{-1}) was attributed to the stretching vibration mode of the amide carbonyl group in polySer. The shift in this peak reflected the structural difference of the amide bonds with specific hydrogen bonds. In particular, the peak top region at 1620−1640 cm^{-1} was assignable to the β-sheet structure.[56] PolyAla and poly[*O*-*tert*-butyl L-serine] [poly(*O*-*tert*-butyl Ser)] prepared via NCA ring-opening polymerization was also reported to form β-structures, suggesting no contribution of the hydroxyl group to the formation of the β-sheet structure, and it was indicated that the propagating polySer formed a β-sheet structure during CEP and assembled into large aggregates, resulting in an insoluble precipitate.

The secondary structure of polySer in aqueous solution was also investigated by CD spectroscopy. An aqueous solution of polySer at various pH values was prepared by using polySer after TFA treatment (Figure S8). After dissolving 1 mg of polySer in deionized water, the pH of the solution was adjusted to 3, 5, 7, 9, and 12 by the addition of NaOH or HCl. The CD

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spectra of polySer at various pH values are displayed in Figure 6b. The CD spectra of polySer in aqueous solution from pH 3 to 12 exhibited a negative Cotton effect with a negative peak at approximately 220 nm and a positive peak at approximately 205 nm, indicating the formation of a β-strand structure. The CD profiles were almost identical regardless of solution pH. This result indicated that polySer strongly tended to form a β-strand structure in aqueous solution even at a basic pH of 12, and this structure enabled polySer to dissolve. Although the TFA treatment might affect the secondary structure of polySer in a solution state, these results coincide with the report that polySer with a number average of a molecular weight of 600 would have a propensity to form a β-strand structure in water.[30]

Subsequently, WAXD analysis of the as-precipitated polySer was performed to investigate the secondary structure. Figure 7 shows the 1D WAXD profile of the as-precipitated polySer obtained by the CEP of Ser-OEt at pH 8.5. Seven strong diffraction peaks were detected and represented *d*-spacings of 2.3, 2.7, 2.9, 3.5, 4.1, 4.6, and 5.4 Å. Typical antiparallel β-sheet patterns in X-ray analysis have been reported for various polypeptides, where the interstrand distance is constant at 4.6−4.7 Å among the polypeptides due to the strong interaction of multiple hydrogen bonds between polypeptide chains.[57−59] The sheet was assembled with fully extended polypeptide chains with the side chains of adjacent amino acids pointing in opposite directions, where the distance between amino acids was 3.5−3.7 Å.[7,59] Thus, the peaks with *d*-spacings of 4.6 and 3.5 Å were assigned to the interstrand distance of polySer and the distance between the serine residues in polySer, respectively (Figure 7b). Moreover, the intersheet distance of a β-sheet structure is known to alter depending on the side chain of the amino acids and is correlated with the van der Waals volume of the amino acids.[57−59] Previously, we also demonstrated that the WAXD 1D profile of linear polyAla exhibited an antiparallel β-sheet structure and revealed that the intersheet distance of the β-sheet structure was 5.2 Å.[33,60,61] The peak with a *d*-spacing of 5.4 Å for polySer was assumed to be the intersheet distance of the β-sheet structure (Figure 7b), and the distance was larger than that of polyAla. The difference in the intersheet distance between polySer and polyAla was caused by the steric repulsion of the hydroxyl group on the side chain of Ser.[57−59] The other diffraction peaks with *d*-spacings of 2.3, 2.7, and 2.9 Å were difficult to assign to a specific periodic structure. However, these peaks were also similar to those appearing in the WAXD profile of polyAla in a β-sheet structure.[33] Collectively, the peaks detected in the WAXD profile of polySer were assumed to be the β-sheet structure, as illustrated in Figure 7b.

CONCLUSIONS

We demonstrated that the protecting group-free synthesis of polySer was achieved by the papain-catalyzed polymerization of Ser-OEt in aqueous solution. At pH 8.5, polySer with DP values ranging from 5 to 22 was obtained as a precipitate after CEP. The MALDI-TOF mass and ^{1}H NMR results of the precipitate revealed that the obtained polySer consisted of a Ser residue with a free hydroxyl group. The precipitated polySer was soluble in TFA or highly basic aqueous solution at pH 12.0. The structural characterization of the obtained polySer using IR spectroscopy, CD spectroscopy, and WAXD indicated that the precipitate was formed via the assembly of the β-sheet structure of polySer during CEP. We are convinced that the facile and sustainable preparation of protecting group-free polySer will lead to the development of versatile functional polypeptides.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acspolymersau.1c00052.

MALDI-TOF MS spectra of the precipitate formed by the CEP after 60 min and ESI MS spectra of the supernatant solution prepared by CEP after 60 min (Figure S1); photographs of the resultant solution after the CEP of Ser-OEt (Figure S2); ^{1}H-^{1}H COSY NMR of the precipitated polySer after the CEP of Ser-OEt (Figure S3); MALDI-TOF MS spectra of the precipitate formed during CEP conducted at different pH values (Figure S4); ESI MS spectra of the supernatant solution prepared by CEP at pH 7.0, 8.5, and 10.0 (Figure S5); ^{1}H-^{1}H COSY NMR of the precipitated polySer after the CEP of Ser-OMe (Figure S6); solubility test of polySer in various solvents (Figure S7); and solubility test of polySer in a TFA/water mixture and its solubilization in water by TFA treatment (Figure S8) (PDF)

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Author Contributions

#T.W. and Kayo T. contributed equally to this paper.

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Conceptualization, K.N. and Kousuke T.; methodology, Kousuke T. and K.N.; analysis, T.W., S.T., Kayo T., and H.M.; writing—original draft preparation, T.W., Kayo T., and Kousuke T.; writing—review and editing, H.M., Kousuke T, and K.N.; visualization, T.W.; supervision, Kousuke T and K.N.; project administration, K.N.; funding acquisition, K.N. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Muttenthaler, M.; King, G. F.; Adams, D. J.; Alewood, P. F. Trends in peptide drug discovery. *Nat. Rev. Drug Discov.* **2021**, *20*, 309.
(2) Numata, K. How to define and study structural proteins as biopolymer materials. *Polym. J.* **2020**, *52*, 1043−1056.
(3) Mondal, S.; Das, S.; Nandi, A. K. A review on recent advances in polymer and peptide hydrogels. *Soft Matter* **2020**, *16*, 1404−1454.
(4) Drucker, D. J. Advances in oral peptide therapeutics. *Nat. Rev. Drug Discov.* **2020**, *19*, 277−289.
(5) Ge, C.; Ye, H.; Wu, F.; Zhu, J.; Song, Z.; Liu, Y.; Yin, L. Biological applications of water-soluble polypeptides with ordered secondary structures. *J. Mater. Chem. B* **2020**, *8*, 6530−6547.
(6) Nishigami, H.; Kang, J.; Terada, R.-I.; Kino, H.; Yamasaki, K.; Tateno, M. Is it possible for short peptide composed of positively- and negatively-charged "hydrophilic" amino acid residue-clusters to form metastable "hydrophobic" packing? *Phys. Chem. Chem. Phys.* **2019**, *21*, 9683−9693.
(7) Bonduelle, C. Secondary structures of synthetic polypeptide polymers. *Polym. Chem.* **2018**, *9*, 1517−1529.
(8) Lee, H.-M.; Ren, J.; Tran, K. M.; Jeon, B.-M.; Park, W.-U.; Kim, H.; Lee, K. E.; Oh, Y.; Choi, M.; Kim, D.-S.; Na, D. Identification of efficient prokaryotic cell-penetrating peptides with applications in bacterial biotechnology. *Commun. Biol.* **2021**, *4*, 205.
(9) Levin, A.; Hakala, T. A.; Schnaider, L.; Bernardes, G. J. L.; Gazit, E.; Knowles, T. P. J. Biomimetic peptide self-assembly for functional materials. *Nat. Rev. Chem.* **2020**, *4*, 615−634.
(10) Adler-Abramovich, L.; Arnon, Z. A.; Sui, X.; Azuri, I.; Cohen, H.; Hod, O.; Kronik, L.; Shimon, L. J. W.; Wagner, H. D.; Gazit, E. Bioinspired flexible and tough layered peptide crystals. *Adv. Mater.* **2018**, *30*, No. 1704551.
(11) Tang, H.; Yin, L.; Lu, H.; Cheng, J. Water-soluble poly(L-serine)s with elongated and charged side-chains: synthesis, conformations, and cell-penetrating properties. *Biomacromolecules* **2012**, *13*, 2609−2615.
(12) Serban, M. A.; Kaplan, D. L. pH-Sensitive ionomeric particles obtained via chemical conjugation of silk with poly(amino acid)s. *Biomacromolecules* **2010**, *11*, 3406−3412.
(13) Love, C. J.; Serban, B. A.; Katashima, T.; Numata, K.; Serban, M. A. Mechanistic insights into silk fibroin's adhesive properties via chemical functionalization of serine side chains. *ACS Biomater. Sci. Eng.* **2019**, *5*, 5960−5967.
(14) António, J. P. M.; Russo, R.; Carvalho, C. P.; Cal, P. M. S. D.; Gois, P. M. P. Boronic acids as building blocks for the construction of therapeutically useful bioconjugates. *Chem. Soc. Rev.* **2019**, *48*, 3513−3536.
(15) Zhang, Y.; Xu, C.; Lam, H. Y.; Lee, C. L.; Li, X. Protein chemical synthesis by serine and threonine ligation. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 6657−6662.
(16) Trader, D. J.; Carlson, E. E. Chemoselective hydroxyl group transformation: an elusive target. *Mol. BioSyst.* **2012**, *8*, 2484.
(17) Alpha-Bazin, B.; Quéméneur, E. Antibody-free detection of phosphoserine/threonine containing peptides by homogeneous time-resolved fluorescence. *Anal. Chem.* **2012**, *84*, 9963−9970.
(18) Cortes-Clerget, M.; Lee, N. R.; Lipshutz, B. H. Synthetic chemistry in water: applications to peptide synthesis and nitro-group reductions. *Nat. Protoc.* **2019**, *14*, 1108−1129.
(19) Bayer, E.; Mutter, M. Liquid phase synthesis of peptides. *Nature* **1972**, *237*, 512−513.
(20) Coin, I.; Beyermann, M.; Bienert, M. Solid-phase peptide synthesis: from standard procedures to the synthesis of difficult sequences. *Nat. Protoc.* **2007**, *2*, 3247−3256.
(21) Merrifield, R. B. Solid phase synthesis (Nobel lecture). *Angew. Chem., Int. Ed.* **1985**, *24*, 799−810.
(22) Wibowo, S. H.; Sulistio, A.; Wong, E. H. H.; Blencowe, A.; Qiao, G. G. Polypeptide films via *N*-carboxyanhydride ring-opening polymerization (NCA-ROP): past, present and future. *Chem. Commun.* **2014**, *50*, 4971.
(23) Habraken, G. J. M.; Heise, A.; Thornton, P. D. Block copolypeptides prepared by N-carboxyanhydride ring-opening polymerization. *Macromol. Rapid Commun.* **2012**, *33*, 272−286.
(24) Yu, J.; Qian, C.; Zhang, Y.; Cui, Z.; Zhu, Y.; Shen, Q.; Ligler, F. S.; Buse, J. B.; Gu, Z. Hypoxia and H_2O_2 dual-sensitive vesicles for enhanced glucose-responsive insulin delivery. *Nano Lett.* **2017**, *17*, 733−739.
(25) Price, D. J.; Khuphe, M.; Davies, R. P. W.; Mclaughlan, J. R.; Ingram, N.; Thornton, P. D. Poly(amino acid)-polyester graft copolymer nanoparticles for the acid-mediated release of doxorubicin. *Chem. Commun.* **2017**, *53*, 8687−8690.
(26) Chen, J.; Guo, Z.; Lin, L.; Hu, Y.; Tian, H.; Chen, M.; Chen, X. Combination therapy of pDNA and siRNA by versatile carriers composed of poly(L-serine) modified polyethylenimines. *Mater. Chem. Front.* **2017**, *1*, 937−946.
(27) Khuphe, M.; Mukonoweshuro, B.; Kazlauciunas, A.; Thornton, P. D. A vegetable oil-based organogel for use in pH-mediated drug delivery. *Soft Matter* **2015**, *11*, 9160−9167.
(28) Lee, H.; Park, J. B.; Chang, J. Y. Synthesis of poly(ethylene glycol)/polypeptide/poly(D,L -lactide) copolymers and their nanoparticles. *J. Polym. Sci., Part A: Polym. Chem.* **2011**, *49*, 2859−2865.
(29) Tooney, N. M.; Fasman, G. D. Synthesis of poly-*O*-*tert*-butyl-L-serine and poly-L-serine. *Biopolymers* **1968**, *6*, 81−96.
(30) Tooney, N. M.; Fasman, G. D. Conformation of serine polypeptides. *J. Mol. Biol.* **1968**, *36*, 355−369.
(31) Bohak, Z.; Katchalski, E. Synthesis, Characterization, and racemization of poly-L-serine*. *Biochemistry* **1963**, *2*, 228−237.
(32) Yang, Z.; Mao, Z.; Ling, J. Phosgene-free synthesis of non-ionic hydrophilic polyserine. *Polym. Chem.* **2016**, *7*, 519−522.
(33) Tsuchiya, K.; Kurokawa, N.; Gimenez-Dejoz, J.; Gudeangadi, P. G.; Masunaga, H.; Numata, K. Periodic introduction of aromatic units in polypeptides via chemoenzymatic polymerization to yield specific secondary structures with high thermal stability. *Polym. J.* **2019**, *51*, 1287−1298.
(34) Yazawa, K.; Gimenez-Dejoz, J.; Masunaga, H.; Hikima, T.; Numata, K. Chemoenzymatic synthesis of a peptide containing nylon monomer units for thermally processable peptide material application. *Polym. Chem.* **2017**, *8*, 4172−4176.
(35) Tsuchiya, K.; Numata, K. Chemoenzymatic synthesis of polypeptides for use as functional and structural materials. *Macromol. Biosci.* **2017**, *17*, No. 1700177.

https://doi.org/10.1021/acspolymersau.1c00052
ACS Polym. Au 2022, 2, 147−156

(36) Tsuchiya, K.; Numata, K. Chemoenzymatic synthesis of polypeptides containing the unnatural amino acid 2-aminoisobutyric acid. *Chem. Commun.* **2017**, *53*, 7318−7321.
(37) Tsuchiya, K.; Numata, K. Papain-catalyzed chemoenzymatic synthesis of telechelic polypeptides using bis(leucine ethyl ester) initiator. *Macromol. Biosci.* **2016**, *16*, 1001−1008.
(38) Ageitos, J. M.; Baker, P. J.; Sugahara, M.; Numata, K. Proteinase K-catalyzed synthesis of linear and star oligo(L-phenylalanine) conjugates. *Biomacromolecules* **2013**, *14*, 3635−3642.
(39) Ageitos, J. M.; Yazawa, K.; Tateishi, A.; Tsuchiya, K.; Numata, K. The Benzyl ester group of amino acid monomers enhances substrate affinity and broadens the substrate specificity of the enzyme catalyst in chemoenzymatic copolymerization. *Biomacromolecules* **2016**, *17*, 314−323.
(40) Fagerland, J.; Finne-Wistrand, A.; Numata, K. Short One-Pot Chemo-enzymatic synthesis of L-lysine and L-alanine diblock co-oligopeptides. *Biomacromolecules* **2014**, *15*, 735−743.
(41) Gudeangadi, P. G.; Tsuchiya, K.; Sakai, T.; Numata, K. Chemoenzymatic synthesis of polypeptides consisting of periodic di- and tri-peptide motifs similar to elastin. *Polym. Chem.* **2018**, *9*, 2336−2344.
(42) Gimenez-Dejoz, J.; Tsuchiya, K.; Numata, K. Insights into the stereospecificity in papain-mediated chemoenzymatic polymerization from quantum mechanics/molecular mechanics simulations. *ACS Chem. Biol.* **2019**, *14*, 1280−1292.
(43) Gimenez-Dejoz, J.; Tsuchiya, K.; Tateishi, A.; Motoda, Y.; Kigawa, T.; Asano, Y.; Numata, K. Computational study on the polymerization reaction of d-aminopeptidase for the synthesis of d-peptides. *RSC Adv.* **2020**, *10*, 17582−17592.
(44) Tsuchiya, K.; Numata, K. Facile terminal functionalization of peptides by protease-catalyzed chemoenzymatic polymerization toward synthesis of polymeric architectures consisting of peptides. *Polym. Chem.* **2020**, *11*, 560−567.
(45) Yazawa, K.; Numata, K. Papain-catalyzed synthesis of polyglutamate containing a nylon monomer unit. *Polymer* **2016**, *8*, 194.
(46) Ma, Y.; Sato, R.; Li, Z.; Numata, K. Chemoenzymatic synthesis of oligo(L-cysteine) for use as a thermostable bio-based material. *Macromol. Biosci.* **2016**, *16*, 151−159.
(47) Ma, Y.; Li, Z.; Numata, K. Synthetic short peptides for rapid fabrication of monolayer cell sheets. *ACS Biomater. Sci. Eng.* **2016**, *2*, 697−706.
(48) Numata, K.; Baker, P. J. Synthesis of adhesive peptides similar to those found in blue mussel (*mytilus edulis*) using papain and tyrosinase. *Biomacromolecules* **2014**, *15*, 3206−3212.
(49) Narai-Kanayama, A.; Hanaishi, T.; Aso, K. α-Chymotrypsin-catalyzed synthesis of poly-l-cysteine in a frozen aqueous solution. *J. Biotechnol.* **2012**, *157*, 428−436.
(50) Fukuoka, T.; Tachibana, Y.; Tonami, H.; Uyama, H.; Kobayashi, S. Enzymatic polymerization of tyrosine derivatives. peroxidase- and protease-catalyzed synthesis of poly(tyrosine)s with different structures. *Biomacromolecules* **2002**, *3*, 768−774.
(51) Numata, K.; Masunaga, H.; Hikima, T.; Sasaki, S.; Sekiyama, K.; Takata, M. Use of extension-deformation-based crystallisation of silk fibres to differentiate their functions in nature. *Soft Matter* **2015**, *11*, 6335−6342.
(52) Lowe, G.; Yuthavong, Y. pH-dependence and structure−activity relationships in the papain-catalysed hydrolysis of anilides. *Biochem. J.* **1971**, *124*, 117−122.
(53) Romero-Montero, A.; Aguirre-Díaz, I. S.; Puiggalí, J.; del Valle, L. J.; Gimeno, M. Self-assembly of supramolecular chemoenzymatic poly-l-phenylalanine. *Polym. Chem.* **2021**, *12*, 1199−1209.
(54) Matsui, T.; Baba, T.; Kamiya, K.; Shigeta, Y. An accurate density functional theory based estimation of pKa values of polar residues combined with experimental data: from amino acids to minimal proteins. *Phys. Chem. Chem. Phys.* **2012**, *14*, 4181−4187.
(55) Gaussier, H. L. N.; Morency, H. L. N.; Lavoie, M. C.; Subirade, M. Replacement of yrifluoroacetic acid with HCl in the hydrophobic purification steps of pediocin PA-1: a structural effect. *Appl. Environ. Microbiol.* **2002**, *68*, 4803−4808.
(56) Huang, W.; Krishnaji, S.; Tokareva, O. R.; Kaplan, D.; Cebe, P. Influence of water on protein transitions: morphology and secondary structure. *Macromolecules* **2014**, *47*, 8107−8114.
(57) Brandt, G. S. Secondary Structure. In *Molecular Life Sciences*, Wells, R. D.; Bond, J. S.; Klinman, J.; Masters, B. S. S., Eds.; Springer: New York, 2018.
(58) Knowles, T. P. J.; Vendruscolo, M.; Dobson, C. M. The amyloid state and its association with protein misfolding diseases. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 384−396.
(59) Fändrich, M.; Dobson, C. M. The behaviour of polyamino acids reveals an inverse side chain effect in amyloid structure formation. *EMBO J.* **2002**, *21*, 5682−5690.
(60) Tsuchiya, K.; Ishii, T.; Masunaga, H.; Numata, K. Spider dragline silk composite films doped with linear and telechelic polyalanine: Effect of polyalanine on the structure and mechanical properties. *Sci. Rep.* **2018**, *8*, 3654.
(61) Tsuchiya, K.; Numata, K. Chemical synthesis of multiblock copolypeptides inspired by spider dragline silk proteins. *ACS Macro Lett.* **2017**, *6*, 103−106.

https://doi.org/10.1021/acspolymersau.1c00052
ACS Polym. Au 2022, 2, 147−156