Poly- α -Glutamic Acid Synthesis Using a Novel Catalytic Activity of RimK from *Escherichia coli* K-12[†]

Kuniki Kino,* Toshinobu Arai, and Yasuhiro Arimura

Department of Applied Chemistry, Faculty of Science and Engineering, Waseda University,
3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555, Japan

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Poly-L- α -amino acids have various applications because of their biodegradable properties and biocompatibility. Microorganisms contain several enzymes that catalyze the polymerization of L-amino acids in an ATP-dependent manner, but the products from these reactions contain amide linkages at the side residues of amino acids: e.g., poly- γ -glutamic acid, poly- ϵ -lysine, and cyanophycin. In this study, we found a novel catalytic activity of RimK, a ribosomal protein S6-modifying enzyme derived from *Escherichia coli* K-12. This enzyme catalyzed poly- α -glutamic acid synthesis from unprotected L-glutamic acid (Glu) by hydrolyzing ATP to ADP and phosphate. RimK synthesized poly- α -glutamic acid of various lengths; matrix-assisted laser desorption/ionization–time of flight–mass spectrometry showed that a 46-mer of Glu (maximum length) was synthesized at pH 9. Interestingly, the lengths of polymers changed with changing pH. RimK also exhibited 86% activity after incubation at 55°C for 15 min, thus showing thermal stability. Furthermore, peptide elongation seemed to be catalyzed at the C terminus in a stepwise manner. Although RimK showed strict substrate specificity toward Glu, it also used, to a small extent, other amino acids as C-terminal substrates and synthesized heteropeptides. In addition, RimK-catalyzed modification of ribosomal protein S6 was confirmed. The number of Glu residues added to the protein varied with pH and was largest at pH 9.5.

Poly-L-amino acids possess biodegradable properties and are therefore useful in various fields, including food science, medicine, and cosmetics. Polyaspartic acid is used as a biodegradable substitute for synthetic polyacrylate (25), and poly- α -glutamic acid finds application in a wide variety of surgical and pharmaceutical products (e.g., for enhancement of solubility and control of half-life of drugs) (27). Furthermore, L-arginine (Arg)-rich peptides, which can permeate cell membranes, are used for intracellular delivery of macromolecules (7). In addition, unique polyamino acids produced by various microorganisms, including poly- γ -glutamic acid, poly- ϵ -lysine, and cyanophycin (multi-L-arginyl-poly[L-aspartic acid]), have been well studied and widely applied (6, 21, 26). These acids have a γ -, ϵ -, or β -amide linkage. Both poly- γ -glutamic acid and poly- ϵ -lysine have characteristic abilities of high water absorbency and antimicrobial activity, respectively, and are therefore used on a commercial scale. Previous studies have revealed the biosynthetic mechanisms of these polyamino acids and have achieved their mass production (5, 33). In contrast, poly-L-amino acids with only an α -amide linkage have not been found in microorganisms, probably because poly-L- α -amino acids might be hydrolyzed by proteases and peptidases contained in the microorganisms. Therefore, poly-L- α -amino acids may not be detectable even in organisms containing an enzyme possessing poly-L- α -amino acid-synthesizing activity.

To create a supply of useful poly- α -amino acids, their po-

tential production by chemical and enzymatic synthesis has been attempted. Chemical synthesis usually involves ring-opening polymerization of α -amino acid *N*-carboxylic anhydrides (27), but this method is not efficient as it employs organic solvent and protecting groups. Some studies have investigated enzymatic synthesis by using proteases. Although proteases primarily catalyze the hydrolysis of proteins and peptides, they also catalyze the formation of peptide bonds under selected conditions (17). Previous research has also shown polymerization of ester hydrochlorides of L-methionine (Met), L-phenylalanine (Phe), L-threonine (Thr), L-tyrosine (Tyr), and L-glutamic acid (Glu), which were used as substrates in a buffer (1, 11, 28, 32). Papain catalyst selectively polymerized L-form amino acid derivatives, but produced shorter peptides (less than 10 residues) (32). Since enzymatic synthesis is an eco-friendly process and can be quite useful, the issues of short peptide length and the use of protecting groups should be further investigated.

We have previously examined peptide synthesis by using ligases (of the EC 6.3.2 class) and have reported novel enzymes and their corresponding catalytic activities (2, 3, 14–16). We found that some L-amino acid ligases (EC 6.3.2.28) catalyzed oligopeptide synthesis (4). L-Amino acid ligase synthesizes various peptides from unprotected amino acids by the hydrolysis of ATP to ADP and phosphate (P_i) (30). Free L-valine (Val), L-leucine (Leu), L-isoleucine (Ile), Met, Phe, L-tryptophan (Trp), and Tyr were combined to generate their oligopeptides and 2- to 8-mers; the lengths of peptides were found to depend on the enzymes used in the reactions (4).

In more recent studies, we focused on RimK, which catalyzes the modification of the ribosomal protein S6 by adding up to 4 Glu residues to the C-terminal sequence of L-aspartic acid (Asp)-L-serine (Ser)-Glu-Glu with α -amide linkage in *Escherichia coli*, and generated Asp-Ser-Glu-Glu-Glu, Asp-Ser-Glu-

* Corresponding author. Mailing address: Department of Applied Chemistry, Faculty of Science and Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555, Japan. Phone: 81-3-5286-3211. Fax: 81-3-3232-3889. E-mail: kkino@waseda.jp.

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Glu-Glu-Glu, Asp-Ser-Glu-Glu-Glu-Glu-Glu, and Asp-Ser-Glu-Glu-Glu-Glu-Glu-Glu sequences (added Glu residues are underlined) (13). In addition, Isono et al. reported that multicopies of *rimK* contributed to the efficiency of the Glu-adding reaction and experimentally confirmed that approximately a 15-mer of Glu was added to the C terminus of ribosomal protein S6 *in vivo* (13). The ribosomal protein S6 is a part of the 30S subunit, but the role of its modification remains unclear (12). RimK also belongs to the ATP-dependent carboxylate-amine/thiol ligase superfamily, similar to L-amino-acid ligase, glutathione synthetase, and cyanophycin synthetase (8); therefore, RimK seems to catalyze ligation in an ATP-dependent manner by producing an aminoacyl-phosphate as a reaction intermediate. In the present study, we identified a novel catalytic activity of RimK: RimK catalyzed the synthesis of poly- α -glutamic acid via ATP hydrolysis from unprotected Glu, in addition to modifying ribosomal protein S6. We also examined several characteristics of RimK by using various methods.

MATERIALS AND METHODS

Materials. *E. coli* K-12 was purchased from NITE Biological Resource Center (Chiba, Japan). *E. coli* BL21(DE3), pET-21a(+) vector, and pET-28a(+) vector were purchased from Merck (Darmstadt, Germany). Hydroxylamine hydrochloride (NH₂OH; iron free for iron analysis) was obtained from Kanto Chemical (Tokyo, Japan). All other chemicals used in this study are commercially available (as indicated) and of chemically pure grade.

Genetic manipulation and preparation of recombinant proteins. DNA manipulation was performed according to the methods of Sambrook et al., with minor modifications (23). *rimK* and *rpsF* were used to code for the ribosomal S6 modification protein and ribosomal protein S6, respectively. Both genes were amplified from the genomic DNA of *E. coli* K-12 by PCR using the following primers: *rimK*_{sense} (5'-AATTTTCATATGAAAATTGCCATATTGTCCC G-3'; NdeI), *rimK*_{antisense} (5'-TAGAATTCGACACCCGTTTTCAGGC-3'; EcoRI), *rpsF*_{sense} (5'-ATTAAATACATATGCGTCATTACGAAATC-3'; NdeI), and *rpsF*_{antisense} (5'-AAGAATTCCTACTCTTCAGAATCCCC-3'; EcoRI). These PCR fragments were digested with NdeI and EcoRI, and the *rimK* and *rpsF* genes were then ligated into the pET-21a(+) and pET-28a(+) vectors, respectively. The resulting plasmids were designed to express the genes with a His tag sequence under the control of the T7 promoter, and each plasmid was then introduced into *E. coli* BL21(DE3).

E. coli BL21(DE3) cells harboring the *rimK*-ligated pET-21a(+) vector were cultivated in 3 ml of Luria-Bertani medium (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl) containing 100 μ g/ml of ampicillin (final concentration). *E. coli* BL21(DE3) cells harboring the *rpsF*-ligated pET-28a(+) were cultivated in the same medium containing 30 μ g/ml of kanamycin (final concentration) at 37°C for 5 h with shaking at 160 rpm. The cultivated cells were transferred to 100 ml of fresh Luria-Bertani medium containing the same antibiotics used during precultivation and were further cultivated at 37°C for 1 h with shaking at 120 rpm. Isopropyl- β -D-thiogalactopyranoside (final concentration, 0.1 mM) was then added, and cultivation was continued at 25°C for 19 h with shaking at 120 rpm. The cells were harvested by centrifugation (4,160 $\times$ g, 10 min, 4°C), resuspended in 100 mM Tris-HCl buffer (pH 8), and then disrupted by sonication at 4°C. Cellular debris was removed by centrifugation (20,000 $\times$ g, 30 min, 4°C), and the supernatant was collected and purified with a HisTrap HP Ni-affinity column (GE Healthcare, Buckinghamshire, United Kingdom). The fractions were then desalted with a PD-10 column (GE Healthcare) and equilibrated with 100 mM Tris-HCl buffer (pH 9). To confirm the presence of protein in the solution, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by Laemmli's method (18). The protein concentration was then determined by the Bradford method, with bovine serum albumin as the standard.

Characterization of RimK. The peptide synthesizing activity of RimK was assayed as follows, unless otherwise specified. The standard reaction mixture used for assays (0.3-ml total volume) contained 25 mM L-amino acid substrate, 12.5 mM ATP, 12.5 mM MgSO₄ · 7H₂O, and 0.5 mg/ml of RimK in 100 mM Tris-HCl buffer (pH 9). The reaction was performed at 30°C for 20 h, and the following standard amino acids and peptides were examined: Arg, L-lysine (Lys), L-histidine (His), L-glutamine (Gln), L-asparagine (Asn), Glu, Asp,

L-alanine (Ala), Ser, Thr, glycine (Gly), L-proline (Pro), Val, Leu, Ile, Met, L-cysteine (Cys), Phe, Trp, Tyr, L-glutamyl-L-glutamic acid (Glu-Glu), L-aspartyl-L-aspartic acid (Asp-Asp), and L-arginyl-L-glutamic acid (Arg-Glu). When reactions involved 2 substrates, the concentration of each substrate was 12.5 mM. To detect the activity of the reactions, the amount of P_i produced in a reaction mixture was determined with a Determiner L IP kit (Kyowa Medex, Tokyo, Japan) according to the manufacturer's protocol. To confirm peptide synthesis, the reaction mixtures were analyzed by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI MS). The reaction product was purified by the ethanol precipitation method, and the resulting products were analyzed by nuclear magnetic resonance (NMR).

To examine the thermal stability of the protein, RimK was incubated at 30, 35, 40, 45, 50, 55, and 60°C prior to initiation of the reactions. To examine the effect of pH, reactions were performed in 100 mM Tris-HCl buffer maintained at pHs 7, 8, 9, 9.5, and 10 for both 1 and 20 h; the reaction mixtures (total volume, 0.3 ml) contained 12.5 mM Glu, 12.5 mM ATP, 12.5 mM MgSO₄ · 7H₂O, and 0.5 mg/ml of RimK. The reaction products were purified by the ethanol precipitation method, and the resulting products were subsequently analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

To determine the molecular mass of the protein, a HiLoad 16/60 Superdex 200-pg column was used (GE Healthcare). The column was equilibrated with 100 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl, and the protein was eluted with the same buffer at a flow rate of 0.5 ml/min.

Dipeptide-activating activity was also assayed by the method of Stulberg et al., with minor modifications (29). The reaction mixture (total volume, 0.3 ml) contained 20 mM Glu-Glu or Asp-Asp, 200 mM NH₂OH, 30 mM ATP, 30 mM MgSO₄ · 7H₂O, and 0.5 mg/ml of RimK in 100 mM Tris-HCl buffer (pH 9). The reaction was performed at 30°C for 20 h. After the reaction, 150 μ l of the reaction mixture was separated, and 75 μ l of 8% trichloroacetic acid and 75 μ l of 3.4% FeCl₃ (dissolved in 2N HCl) were added. The precipitate was then removed by centrifugation (20,000 $\times$ g, 30 min, 4°C). The supernatant was collected, and its absorbance (at 490 nm) was measured with a microplate reader (Bio-Rad model 550; Bio-Rad Laboratories, Hercules, CA).

Modification of ribosomal protein S6 (RpsF) was examined as follows, unless otherwise specified. The reaction mixture (total volume, 0.3 ml) contained 20 mM Glu, 20 mM ATP, 20 mM MgSO₄ · 7H₂O, 0.5 mg/ml of RpsF, and 0.5 mg/ml of RimK in 100 mM Tris-HCl buffer (pH 9). The reaction was performed at 30°C for 20 h. Reactions with 20 mM Glu-Glu substrate were also conducted. The pH was changed, and reactions were performed in 100 mM Tris-HCl buffer maintained at pHs 9, 9.5, and 10. The reaction products were analyzed by the previously described tricine-SDS-PAGE method with minor modifications (24).

Analysis of peptides. (i) **Ethanol precipitation.** The reaction mixture was boiled for 10 min and then centrifuged (20,000 $\times$ g, 30 min, 4°C) to remove precipitates. The resulting supernatant was added to 1/5 volume of 5 M NaCl and 2 volumes of 100% ethanol. The mixture was thoroughly mixed, incubated at -80°C for 20 min, and then centrifuged (20,000 $\times$ g, 30 min, 4°C). The supernatant was then removed, and the precipitate was recovered as the purified reaction product.

(ii) **LC-ESI MS.** The reaction mixtures were centrifuged (20,000 $\times$ g, 30 min, 4°C), and the supernatants were analyzed by LC-ESI MS (for high performance liquid chromatography [HPLC], Agilent 1100 series; Agilent Technologies, Santa Clara, CA; for ESI MS, LCQ Deca; Thermo Scientific, Waltham, MA). The details of these analytical procedures are described in our previous publication (16).

(iii) **MALDI-TOF MS.** Each purified reaction product was dissolved in and diluted 10-fold with Milli-Q water. The resulting solutions were spread on target plates coated with α -cyano-4-hydroxycinnamic acid (CHCA) as the matrix. The plates were dried and loaded into MALDI-TOF mass spectrometer (Autoflex III; Bruker Daltonics, Billerica, MA).

(iv) **NMR.** Each purified reaction product was dissolved in D₂O, and ¹H-NMR spectra were acquired with an AVANCE600 NMR spectrometer (Bruker Bio-Spin, Rheinstetten, Germany). Tetramethylsilane (TMS) was used as an external standard. In addition, poly-L-glutamic acid sodium salt (molecular weight, 750 to 5,000; Sigma, St. Louis, MO), poly- γ -glutamic acid (molecular weight, 200,000 to 500,000; Wako, Osaka, Japan), and γ -L-glutamyl-L-glutamate were analyzed.

Nucleotide sequence accession numbers. The nucleotide sequences of the *rimK* and *rpsF* genes have been submitted to GenBank under accession no. X15859.1 and X04022.1, respectively.

FIG. 1. Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI MS) analysis of the RimK-catalyzed reaction. The LC-ESI MS spectrum was obtained at retention times of 5.85 to 5.93 min. Peptides are shown in brackets as EX, where E represents Glu and X is the peptide length. For example, E5 indicates a 5-mer of Glu, and E10 indicates a 10-mer of Glu. The peaks were assigned as follows: m/z 664.2, $[E5 + H]^+$; m/z 793.3, $[E6 + H]^+$; m/z 922.3, $[E7 + H]^+$; m/z 1,051.4, $[E8 + H]^+$; m/z 1,180.4, $[E9 + H]^+$; m/z 1,309.4, $[E10 + H]^+$; and m/z 1,438.5, $[E11 + H]^+$.

RESULTS

Peptide synthesis using RimK and identification of reaction products. We hypothesized that RimK exhibits a different catalytic activity, such as peptide synthesis, when unprotected acidic amino acids or acidic peptides are used as substrates. To test this hypothesis, RimK was prepared as a C-terminal His-tagged protein, and its ability to catalyze peptide synthesis was subsequently investigated. First, homopeptide synthesis was examined by using 20 proteogenic amino acids, Glu-Glu, and Asp-Asp. Since RimK causes the hydrolysis of ATP to ADP and P_i during peptide bond formation, the amount of P_i produced in the reaction mixture was measured as the first screening for the detection of ligase activity. In our previous studies, we found that ligase enzymes produce some P_i even in the absence of an amino acid substrate. Hence, the negative control was the reaction carried out in the absence of amino acids, and the ligase activity was judged by the detection of P_i in amounts greater than this blank value (2.3 ± 0.13 mM; average of 2 measurements). Interestingly, a significant amount of P_i (10.2 ± 0.09 mM; average of 2 measurements) was detected only when Glu was used; Glu-Glu, Asp, and Asp-Asp were not recognized as substrates. As the next step, the reaction mixture containing Glu was analyzed by LC-ESI MS: 5- to 11-mers of Glu were detected at a retention time of 5.85 to 5.93 min (Fig. 1), and up to 15-mer homopolymers (m/z 1,954.7, $[E15 + H]^+$) were additionally detected at a retention time of 5.93 to 6.10 min (data not shown). LC-ESI MS analysis suggested that RimK catalyzed poly-glutamic acid synthesis, but it was unclear whether these polymers had an α - or γ -amide linkage. Thus, the reaction products were partially purified by the ethanol precipitation method, and the resulting products were analyzed by NMR (Fig. 2). The 1.7- to 2.5-ppm region was enlarged, and each NMR chart showed a distinctive waveform between α - and γ -amide linkages. The peaks of the reaction product at 1.8 ppm, 1.9 ppm, and 2.1 to 2.2 ppm (Fig. 2A) were identical to those of poly- α -glutamic acid (Fig. 2B); hence, we

concluded that the reaction product had α -amide linkage. Taken together, RimK catalyzes poly- α -glutamic acid synthesis from unprotected Glu in an ATP-dependent manner.

Characterization of RimK reaction. The effects of temperature and pH on peptide synthesis activity of RimK were investigated. To examine thermal stability, peptide synthesis was performed after RimK was incubated at 20 to 60°C for 15 min, and its activity was subsequently measured by assessing the level of P_i produced in the reaction mixtures (Fig. 3A). RimK exhibited 86% activity when incubated at 55°C, but its activity decreased sharply at 60°C. To examine the effect of pH, the reactions were performed at various pHs, and the activity was assayed by measuring P_i production (Fig. 3B). The largest amount of P_i was detected at pH 9.5. When the reaction was performed for 20 h, 6.2 mM P_i was released into the reaction mixture. The amount of P_i at pHs 9 and 10 was similar to that at pH 9.5.

LC-ESI MS and MALDI-TOF MS analyses were also performed for various pH treatments in order to determine the lengths of the resultant peptides. LC-ESI MS analysis showed that poly- α -glutamic acid was synthesized in all reaction mixtures (data not shown). Interestingly, MALDI-TOF MS analysis revealed that the maximum length of the polymer differed with changing pH (Fig. 4). At pH 9, the reaction product showed polydispersity, and 8- to 46-mers of Glu were detected. At pH 9.5, the length of peptides was shorter than that at pH 9, and 8- to 26-mers of Glu were detected. At pH 10, 8- to 12-mers of Glu were detected. The peptide length at pH 10 was the shortest among pH 9, 9.5, and 10 treatments. Furthermore, the signal intensities increased with pH. As described above, similar amounts of P_i were produced at pHs 9, 9.5, and 10, suggesting that the amount of each polymer synthesized at pH 9 was not large, although there was polydispersity. In contrast, the amounts of each polymer synthesized at pHs 9.5 and 10 were larger than that synthesized at pH 9, although the lengths of polymers synthesized at pHs 9.5 and 10 were shorter than

FIG. 2. Determination of the chemical structure of the reaction product by nuclear magnetic resonance (NMR). NMR analysis was performed to determine whether the reaction product had an α -amide linkage or γ -amide linkage of Glu. ^1H -NMR was performed; the NMR charts of 1.7 to 2.5 ppm are shown. (A) Reaction product; (B) poly-L-glutamic acid sodium salt (poly- α -Glu); (C) poly- γ -glutamic acid (poly- γ -Glu); (D) γ -L-glutamyl-L-glutamate (γ -Glu-Glu).

that at pH 9. It is noteworthy that reaction products were also detected when RimK was incubated at pHs 7 and 8 by the same procedure, but MALDI-TOF MS failed to detect the m/z peaks that corresponded to poly- α -glutamic acid.

Substrate specificities of RimK. As described above, homopeptide synthesis showed that RimK had strict substrate specificity toward Glu and that it did not use Glu-Glu, Asp, or Asp-Asp, although all 4 substrates are anionic compounds.

Thus, RimK may not ligate dipeptides to one another. However, since RimK synthesized poly- α -glutamic acid from free Glu, it is reasonable to assume that RimK would use Glu-Glu as the substrate for polymer extension.

Since RimK belongs to the ATP-dependent carboxylate-amine/thiol ligase superfamily, the dipeptide-activating activity of RimK was assayed by detecting dipeptide hydroxamate synthesis. Phosphorylation at the carboxyl group of a dipeptide

FIG. 3. Effects of temperature and pH on the peptide synthesis activity of RimK. (A) Thermal stability of RimK. RimK was incubated at 20 to 60°C for 15 min before being used for peptide synthesis. The activity of the reactions was assessed by measuring phosphate (P_i) generation. (B) Effect of pH on RimK. Reactions were performed in 100 mM Tris-HCl buffer at pHs 7, 8, 9, 9.5, and 10, and the activity was assayed by measuring P_i after incubation for 1 h (open circles) and 20 h (closed circles). Averages of 3 measurements are shown.

substrate leads to the nucleophilic action of hydroxylamine on the resulting aminoacyl-phosphate, thereby yielding dipeptide hydroxamate. This hydroxamate forms a complex with Fe^{3+} , and the dipeptide activation is detected as a red coloration. In

these experiments, the reactions were conducted using Glu-Glu and Asp-Asp as the substrates. The reaction mixture containing Glu-Glu, but not Asp-Asp, showed red coloration; this confirmed that RimK uses Glu-Glu as an N-terminal, not a C-terminal, substrate.

Next, tripeptide synthesis was examined with Glu-Glu and 19 proteogenic amino acids (all except Glu). LC-ESI MS analysis showed that 15 kinds of Glu-Glu-Xaa (where Xaa represents proteogenic amino acids used, except for Arg, Lys, His, and Pro) were detected, although the amounts of P_i produced in the reaction mixtures were much smaller than those produced when Glu was used as the substrate (see Table S1 in the supplemental material). Although RimK shows preference to Glu as a C-terminal substrate, it does interact to some extent with other substrates.

Heteropeptide synthesis was further examined by using Arg-Glu and 20 proteogenic amino acids, including Glu, as substrates. When Arg-Glu and Glu were used as the substrate, LC-ESI MS analysis showed that the m/z peaks corresponded to poly- α -glutamic acid containing Arg at the N terminus (see Fig. S1 in the supplemental material). As expected, homopolymers of Glu were also detected in this reaction mixture. When Arg-Glu and the other 19 proteogenic amino acids were used, the amounts of P_i (less than 0.3 mM) were much smaller than that when Glu-Glu was used, and only traces of m/z peaks that corresponded to Arg-Glu-Ser and Arg-Glu-Ala were detected. These results suggest that substrates containing cationic residues were unsuitable for peptide synthesis using RimK.

In addition, heteropeptide synthesis was examined with Glu and 19 other proteogenic amino acids as substrates. Only Glu polypeptides were detected in the reaction mixtures by LC-ESI MS. As described above, RimK uses various amino acids as the C-terminal substrate; however, these LC-ESI MS data suggest the specific preference of RimK for Glu. Peptide synthesis using D-form proteogenic amino acids showed that these amino acids were not recognized as substrates, because P_i was not detected in their reaction mixtures.

FIG. 4. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) analysis of the reaction catalyzed by RimK at different pHs. The reaction products in 100 mM Tris-HCl buffer at pHs 10 (A), 9.5 (B), and 9 (C) were analyzed by MALDI-TOF MS (a.u., arbitrary units). Charts are shown as stacks, and the numbers of peptide lengths are also shown.

FIG. 5. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the analysis of the modification of ribosomal protein S6 (RpsF) catalyzed by RimK. In panel A, the substrates were changed in each reaction mixture, and reactions were performed at pH 9. In panel B, the pH condition was changed in each reaction mixture, and the reaction mixture contained RpsF and L-glutamic acid (Glu) as substrates. Lanes: M, molecular mass standard; 1, RpsF (negative control); 2, reaction mixture containing RpsF and Glu as substrates; 3, reaction mixture containing RpsF and Glu-Glu as substrates; 4, reaction mixture containing RpsF, Glu, and Glu-Glu as substrates; 5, RpsF (negative control; same as lane 1); 6, pH 9; 7, pH 9.5; and 8, pH 10. The RimK band was observed between 31 and 45 kDa on SDS-PAGE gels.

Modification of ribosomal protein S6 by RimK. Isono et al. previously identified RimK as the enzyme that catalyzes the modification of the ribosomal protein S6 in *E. coli* K-12 (13). In this study, the effect of the addition of Glu residues to recombinant ribosomal protein S6 (RpsF) was examined *in vitro*. RpsF was prepared as an N-terminal His-tagged protein, and the reactions were conducted using Glu and/or Glu-Glu with RpsF as substrates. Modified RpsF samples were analyzed by tricine-SDS-PAGE; specifically, each modification was detected by the presence of a band shift on SDS-PAGE, which was due to a change in molecular weight. As shown in Fig. 5A, RimK catalyzed the addition of Glu residues to RpsF but failed to ligate Glu-Glu to RpsF. Thus, RimK may elongate peptides at the C-terminal end in a stepwise manner. The results of homopeptide synthesis for the initial screening of ligase activity and the Glu-Glu hydroxamate synthesis data also support this idea. When Glu and Glu-Glu were used as substrates, a band shift was observed; however, the change in the molecular weight was smaller than that when only Glu was used (Fig. 5A, lane 4). This is possibly because in addition to modifying RpsF, RimK catalyzed poly- α -glutamic acid synthesis from Glu-Glu and Glu.

We also examined the modification of RpsF at different pHs (9, 9.5, and 10). Band shifts were observed in all reaction conditions, but the change in molecular weight was different for each pH (Fig. 5B). Specifically, the number of Glu residues added to RpsF was the largest at pH 9.5, which was different from the result of poly- α -glutamic acid synthesis, in which the number of Glu residues was the largest at pH 9 (Fig. 4). LC-ESI MS confirmed the synthesis of poly- α -glutamic acid in all reaction mixtures. Thus, the optimal pH condition for homopolymer synthesis and that for modification may be different.

Determination of molecular mass of RimK. The molecular mass of RimK estimated by gel filtration was 137.2 kDa (average of 2 measurements). This result suggests that RimK is a tetrameric enzyme, since the molecular mass calculated on the basis of amino acid sequence was 34.6 kDa.

DISCUSSION

In this study, we found a novel catalytic activity of RimK—catalysis of poly- α -glutamic acid synthesis—in addition to its activity of modifying the ribosomal protein S6. The lengths of poly- α -glutamic acids were up to 46-mer, which was much longer than those of the products obtained by protease polymerization (32). RimK showed interesting catalytic properties upon exposure to a range of pH conditions, and the lengths of reaction products changed with pH. Thus, it may be possible to control the length of polymers by managing pH conditions. In addition, RimK showed thermal stability against incubation at temperatures up to 55°C, which seems like a preferable property for industrial use.

We could not appropriately measure the amounts of products obtained in this study. That is, 40 mg of purified reaction products was obtained (dry weight) by the ethanol precipitation method from 3 ml of reaction mixture, which contained 40 mM Glu, 40 mM ATP, 40 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.0 mg/ml of RimK in 100 mM Tris-HCl buffer at pH 9. However, LC-ESI MS analysis showed that this precipitate was contaminated with ADP; therefore, the actual amount of poly- α -glutamic acids could not be estimated. The copper sulfate precipitation method was used to obtain only poly- α -glutamic acid. As a preliminary test, the copper sulfate precipitation was used for only commercially available poly- α -glutamic acid (Sigma), as described by Margaritis et al. (20). Three types of poly- α -glutamic acids, with molecular weights of 750 to 5,000, 3,000 to 15,000, and 15,000 to 50,000, were examined, but the precipitate was observed only when the poly- α -glutamic acid with a molecular weight of 15,000 to 50,000 was used. It also seemed that the reaction products synthesized by RimK could not be purified by the copper sulfate precipitation method, probably because the maximum molecular weight of the polymer was approximately 6,000. However, since almost all ATP was consumed in the reaction, as shown by the P_i analysis for initial screening of ligase activity, the maximum yield might have been achieved. Therefore, we believe that large amounts of poly- α -glutamic acids can possibly be synthesized by increasing the concentrations of ATP and Glu in the reaction mixture. When the reaction was performed using 25 mM Glu and 12.5 mM ATP, 10.2 mM P_i was detected, but a reaction with about twice the amount of ATP (20 mM) produced about twice the amount of P_i (19.3 mM). Since RimK condensates unprotected amino acid substrate by hydrolyzing ATP, this protein may find application in fermentative methods that use microorganisms overexpressing *rimK* for mass production of poly- α -amino acids, which is thought to be the most economical and eco-friendly manufacturing process (31).

As described above, RimK catalyzes the modification of RpsF by adding Glu residues to the C-terminal sequence of Asp-Ser-Glu-Glu. A BLAST search (10) showed that various bacteria possess proteins homologous to RimK and RpsF. Both proteins are highly conserved in *E. coli*, *Salmonella* spe-

cies, and *Enterobacter* species; however, many species that possess a RimK homolog do not have an RpsF homolog ending in Glu-Glu at the C terminus. LysX from *Thermus thermophilus* and CofF and MptN from *Methanococcus jannaschii* are the reported RimK homologs that have catalytic functions (9, 19). The activity of each homolog was different from that of RimK, although they commonly catalyzed the ligation of one anionic amino acid to their own N-terminal substrates, a LysW protein, coenzyme γ -F₄₂₀-2, and tetrahydromethanopterin. All homologs shared approximately 30% homology with RimK in terms of their amino acid sequences, and CofF was determined to be farthest from RimK in the phylogenetic tree (19). Using recombinant LysX and MptN, we examined their polymerization activity by the same method used for RimK. The reactions were conducted using 20 proteogenic amino acids as substrates, but a significant amount of P_i was not detected in any of these reaction mixtures (data not shown), suggesting that the polymerization activity we observed is a unique property of RimK.

The novel RimK activity detected in this study is particularly important for developing methods of enzymatic poly- α -amino acid synthesis, especially because it is difficult to obtain enzymes that synthesize poly- α -amino acids by screening microorganisms, which may contain various peptidases and proteases. Along these lines, we are currently examining the alteration of substrate specificity and the enhancement of the catalytic activity of RimK by applying evolutionary engineering methods. However, the crystal structure of RimK has not been elucidated experimentally but has been predicted by a homology modeling method using a crystal structure of LysX as the template (22; data not shown). This information may be a powerful tool for generating new enzymes that synthesize novel poly- α -amino acids. We further expect that RimK is applicable to protein modification by virtue of its property of adding poly- α -glutamic acid tails to various proteins. Addition of such anionic residues to proteins may enhance their solubility and render them useful as affinity tags, although more investigations are necessary to examine their practical use. We are hopeful that this study on RimK will serve as a breakthrough in poly- α -amino acid synthesis.

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