Poly-α-Glutamic Acid Synthesis Using a Novel Catalytic Activity of RimK from *Escherichia coli K-12* \(^{\dagger}\)

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Poly-L-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.

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Materials. E. coli K-12 was purchased from NITE Biological Resource Center (Chiba, Japan). E. coli BL21(DE3) vector, 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 genes 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’-AATTTTCCATATGAAAAATTCATATGGCC G-3′; NdeI), rimK antisense (5’-TAGATGCGGACCCGCTTTCGACG-3′; EcoRI), rpsF sense (5’-ATTTAATACATGCGTATTACGAAATC-3′; NdeI), and rpsF antisense (5’-AAGAATCTTACCTAGAATCC-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 as a His tag sequence under the control of the T7 promoter, and each plasmid vector was then introduced into E. coli BL21(DE3).

Characterization of RimK. The peptide synthesizing the 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 7). The reaction was performed at 30°C for 20 h. Reactions with 20 mM Glu-Glu substrate were 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, 10, and 10. The reaction products were analyzed by 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 × g, 30 min, 4°C) to remove precipitates. The resulting supernatant was added to 1/5 volume of 5 M NaCl and centrifuged (20,000 × g, 30 min, 4°C). The supernatant was then removed, and the precipitate was recovered by the Bradford method, with bovine serum albumin as the standard.

Analysis of RimK. The 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, 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 200-7.5 cm polished 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.

Peptide-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 NH₄OH, 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 μl of the reaction mixture was subjected to polyacrylamide gel electrophoresis (SDS-PAGE). TLC analysis was then performed, and the absorbance (at 405 nm) was measured with a microplate reader.

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, 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. The reaction mixture was separated, and 75 μl of 8% trichloroacetic acid and 75 μl of 3.4% FeCl₃ (dissolved in 2N HCl) were added. The precipitate was then removed by centrifugation (20,000 × g, 30 min, 4°C). The supernatant was collected, and its absorbance (at 405 nm) was measured with a microplate reader.

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-Glu or Asp-Asp, 200 mM NH₄OH, 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 μl of the reaction mixture was subjected to polyacrylamide gel electrophoresis (SDS-PAGE). TLC analysis was then performed, and the absorbance (at 405 nm) was measured with a microplate reader.
we found that ligase enzymes produce some Pi 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 its activity was subsequently measured by assessing the amount of Pi production (Fig. 3B). The largest amount of Pi was detected at pH 9.5. When the reaction was performed for 20 h, 6.2 mM Pi was released into the reaction mixture. The amount of Pi at pHs 9 and 10 was similar to that at pH 9.5.

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 Pi 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 Pi production (Fig. 3B). The largest amount of Pi was detected at pH 9.5. When the reaction was performed for 20 h, 6.2 mM Pi was released into the reaction mixture. The amount of Pi 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 Pi 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 8- to 26-mers of 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...
substrate leads to the nucleophilic action of hydroxylamine on the resulting aminoacyl-phosphate, thereby yielding dipeptide hydroxamate. This hydroxamate forms a complex with $\text{Fe}^{3+}$, 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-$\alpha$-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 $\alpha$-form proteogenic amino acids showed that these amino acids were not recognized as substrates, because P$_i$ was not detected in their reaction mixtures.
**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-Glu 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-Glu-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-Glu-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 MgSO₄·7H₂O, 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 Pı 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 Pi was detected, but a reaction with about twice the amount of ATP (20 mM) produced about twice the amount of Pı (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 RpsE homolog ending in Glu-Glu at the C terminus. LysX from Thermus thermophilus and CoIF and MptN from Methanocococcus 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 CoIF 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 Pi 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 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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