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# A Novel L-Amino Acid Ligase from *Bacillus subtilis* NBRC3134, a Microorganism Producing Peptide-Antibiotic Rhizocticin

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**L-Amino acid ligase catalyzes the formation of an α-peptide bond from unprotected L-amino acids in an ATP-dependent manner, and this enzyme is very useful in efficient peptide production. We performed enzyme purification to obtain a novel L-amino acid ligase from *Bacillus subtilis* NBRC3134, a microorganism producing peptide-antibiotic rhizocticin. Rhizocticins are dipeptide or tripeptide antibiotics and commonly possess L-arginyl-L-2-amino-5-phosphono-3-*cis*-pentenoic acid. The purification was carried out by detecting L-arginine hydroxamate synthesis activity, and a target enzyme was finally purified 1,280-fold with 0.8% yield. The corresponding gene was then cloned and designated *rizA*. *rizA* was 1,242 bp and coded for 413 amino acid residues. Recombinant RizA was prepared, and it was found that the recombinant RizA synthesized dipeptides whose N-terminus was L-arginine in an ATP-dependent manner. RizA had strict substrate specificity toward L-arginine as the N-terminal substrate; on the other hand, the substrate specificity at the C-terminus was relaxed.**

**Key words:** L-amino acid ligase; peptide synthesis; rhizocticin

L-Amino acid ligase (EC 6.3.2.28) catalyzes the formation of an α-peptide bond from unprotected L-amino acids in an ATP-dependent manner,[1)] and has an ATP-grasp motif that is a signature of the ATP-dependent carboxylate-amine/thiol ligase superfamily.[2)] YwfE protein from *Bacillus subtilis* 168 has been reported as the first L-amino acid ligase, and it synthesizes various dipeptides.[1)] Thereafter, RSp1486a protein from *Ralstonia solanacearum* JCM10489 and BL00235 protein from *Bacillus licheniformis* NBRC12200 were reported to be L-amino acid ligase.[3,4)] These proteins were obtained by *in silico* screening using an ATP-grasp motif as the one of indexes. The *ywfE* gene is part of the biosynthetic gene cluster of a bacilysin peptide-antibiotic that consists of L-alanine (Ala) and L-anticapsin.[5)] Thus the physiological significance of the YwfE protein lies in its participation in peptide antibiotic biosynthesis. In contrast, the roles of the RSp1486a and BL00235 proteins remain unclear.

L-Amino acid ligase is very useful in efficient peptide production. The YwfE protein has made it possible to develop a fermentative method for L-alanyl-L-glutamine production,[6)] but the variety of dipeptides that are synthesized by the YwfE protein is limited due to enzyme substrate specificity. The RSp1486a and BL00235 proteins that were newly obtained by *in silico* screening have different substrate specificities from that of the YwfE protein,[3,4)] but an L-amino acid ligase that takes positively or negatively charged amino acids as N-terminal substrates has not been found. *In silico* screening is a very useful way to newly find an L-amino acid ligase, but it has two disadvantages: (i) it is difficult to predict the substrate specificity from only the amino acid sequence due to a lack of structural information, and it is unclear until the L-amino acid ligase activity of the recombinant protein is assayed; and (ii) the range of search is limited to the microorganisms whose genomic DNA sequences are determined. Therefore, we needed a different approach to obtain a novel L-amino acid ligase, and we focused on a microorganism producing the peptide-antibiotic rhizocticin.

Rhizocticin is an antifungal phosphono-oligopeptide produced by *Bacillus subtilis* NBRC3134 (= ATCC6633).[7,8)] As to the chemical structure, four types of rhizocticins were reported: rhizocticin A is L-arginyl-L-2-amino-5-phosphono-3-*cis*-pentenoic acid (Arg-APPA); rhizocticin B is L-valyl-L-arginyl-L-2-amino-5-phosphono-3-*cis*-pentenoic acid (Val-Arg-APPA); and rhizocticin C and D are same as rhizocticin B but contain L-isoleucine (Ile) and L-leucine (Leu), respectively in place of Val (Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site).[9)] It is believed that the amino acids bound at its N-terminus are necessary for efficient uptake into the target cell and are cleaved by cellular peptidases, and that the released APPA causes antifungal activity through inhibition of threonine synthase activity.[10–15)] No enzymes or genes involved in rhizocticin biosynthesis have been identified.

Based on a report that the YwfE protein is involved in peptide antibiotic biosynthesis,[1)] we formulated the hypothesis that an L-amino acid ligase plays a role in the formation of α-peptide bonds in rhizocticin biosynthesis. In the present study, we found a novel L-amino acid ligase RizA from *B. subtilis* NBRC3134 by enzyme purification. Furthermore, we cloned *rizA* and determined some characteristics of recombinant RizA.

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## Materials and Methods

*Materials. Bacillus subtilis* NBRC3134 (= ATCC6633) was from the NITE Biological Resource Center (Chiba, Japan). pUC19 vector was purchased from Nippon Gene (Tokyo). *Escherichia coli* Rosetta (DE3) and pET30Xa/LIC Vector Kits were from Merck (Darmstadt, Germany). A *Hin*dIII cassette and a *Pst*I cassette were from Takara Bio (Shiga, Japan). Hydroxylammonium chloride (iron free, for iron analysis) was from Kanto Chemical (Tokyo). All other chemicals used are commercially available and were of chemically pure grade.

*Cultivation of* B. subtilis *NBRC3134 and preparation of cell-free extract.* NBRC No. 802 medium (1% polypeptone, 0.2% yeast extract, 0.1% $MgSO_4 \cdot 7H_2O$) and Production medium (0.1% L-asparagine, 0.5% L-glutamate, 2% mannitol, 0.1% $KH_2PO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.000015% $FeSO_4 \cdot 7H_2O$, 0.000016% $ZnSO_4 \cdot 7H_2O$, 0.0005% $MnSO_4 \cdot 5H_2O$; pH 6.0, adjusted with NaOH) were prepared.[12] First, *B. subtilis* NBRC3134 was cultivated in NBRC No. 802 medium at 30 °C with shaking at 120 rpm (overnight). Next, cultivated cells were transferred to Production medium and cultivated for 48 h at 27 °C with shaking at 120 rpm. The cells were harvested by centrifugation (4,160 × *g*, 10 min, 4 °C), resuspended in 50 mM Tris–HCl buffer (pH 8.0), and then disrupted by sonication at 4 °C. Cellular debris was removed by centrifugation (20,000 × *g*, 30 min, 4 °C), and the supernatant was collected as cell-free extract.

*Assay of L-arginine hydroxamate (Arg-NHOH) synthesis activity.* A reagent mixture containing 20 mM L-arginine (Arg), 200 mM hydroxylammonium chloride ($NH_2OH$), 30 mM ATP, and 30 mM $MgSO_4 \cdot 7H_2O$ in 50 mM Tris–HCl buffer (pH 8.0) was prepared. An enzyme solution was added to the reagent mixture, and this was incubated at 30 °C for 1–20 h. To detect Arg-NHOH, the colorimetric method was used with some modifications, as described below.[16] After the reaction, 150 µl of the reaction mixture was prepared, and 75 µl of 8% trichloroacetic acid and 75 µl of 3.4% $FeCl_3$ (dissolved in 2 N HCl) were added. The precipitant was then removed by centrifugation (20,000 × *g*, 30 min, 4 °C). The supernatant was collected, and the absorbance at 490 nm was measured with a microplate reader (Bio Rad Model 550, Bio-Rad Laboratories, Hercules, CA). To determine enzyme activity, HPLC analysis was performed, and one unit (1 U) of enzyme activity was defined as the amount of enzyme that produces 1 µmol of Arg-NHOH per min. The protein concentration was determined by the Bradford method, with bovine serum albumin as the standard.

*Enzyme purification and protein analyses.* All purification procedures were performed at 4 °C or on ice, and purification was carried out by detecting Arg-NHOH synthesis activity. First, ammonium sulfate, $(NH_4)_2SO_4$, was added to a cell-free extract up to 30% saturation. After being stirred for 1 h, the suspension was centrifuged (20,000 × *g*, 60 min, 4 °C), and the supernatant was brought to 60% saturation with $(NH_4)_2SO_4$. The resulting precipitant (30–60% fraction) was collected by centrifugation (20,000 × *g*, 60 min, 4 °C), and was dissolved in 50 mM Tris–HCl buffer (pH 8.0) containing 0.7 M $(NH_4)_2SO_4$. After centrifugation, the supernatant was loaded onto a HiPrep 16/10 Butyl FF column (GE Healthcare, Buckinghamshire, UK) previously equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 0.7 M $(NH_4)_2SO_4$, and the column was washed with the same buffer. The enzyme was eluted with a linear gradient of 0.7–0 M $(NH_4)_2SO_4$ at a flow rate of 2 ml/min. The active fractions were collected and dialyzed against 50 mM Tris–HCl buffer (pH 8.0). Next, the resulting solution was loaded onto a Mono Q 5/50 GL column (GE Healthcare) previously equilibrated with 50 mM Tris–HCl buffer (pH 8.0), and the column was washed with the same buffer. The enzyme was eluted with a linear gradient of 0–0.7 M NaCl at a flow rate of 1 ml/min. The active fractions were collected and concentrated by ultrafiltration. Finally, the enzyme solution was loaded onto a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) previously equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 0.15 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 0.5 ml/min, and the active fractions were collected and concentrated by ultrafiltration.

To confirm protein purity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli.[17] The N-terminal amino acid sequence of the purified enzyme was determined by Kyowa Hakko Kogyo (Tokyo). The molecular mass was determined with a HiLoad 16/60 Superdex 200 pg column (GE Healthcare). The column was equilibrated with 50 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.

*Cloning of the gene encoding the purified enzyme.* DNA manipulations were performed according to the method of Sambrook *et al.*, with minor modifications.[18] To obtain a DNA sequence of the purified enzyme, the cassette PCR method was used.[19] Genomic DNA of *B. subtilis* NBRC3134 was digested with *Hin*dIII or *Pst*I, and these DNA fragments were ligated to the *Hin*dIII and the *Pst*I cassettes respectively. The resulting DNA fragments were used as templates of PCR amplification, and the following primer-sets were used: C1–P1 and C1–P2 toward the *Hin*dIII fragment, and C1–P3 toward the *Pst*I fragment (Table 1). The PCR fragments were digested with *Hin*dIII or *Pst*I and ligated to pUC19 vector. The resulting plasmid was used for sequence analysis. The DNA fragment amplified using C1–P1 was sequenced using primers V1, V2, S1, and S2; the DNA fragment amplified using C1–P2 was sequenced using primers V1, S3, and S4; and the DNA fragment amplified using C1–P3 was sequenced using primers V1, S5, and S6.

*Overexpression of* rizA *and characterization of RizA.* The gene encoding the purified enzyme was designated *rizA* (DDBJ accession no. AB437349). *rizA* was amplified from genomic DNA of *B. subtilis* NBRC3134 by PCR using primers R1 and R2 (Table 1). The PCR fragment was ligated into the pET30Xa/LIC vector according to the protocol that came with the pET30Xa/LIC Vector Kits. The resulting plasmid, pBsRzcA, was designed to express the gene with an N-terminal His-tag sequence under the control of the T7 promoter, and was introduced into *E. coli* Rosetta(DE3).

First, recombinant *E. coli* cells were cultivated in 3 ml of Luria-Bertani medium (1% bacto tryptone, 0.5% yeast extract, 1% NaCl) that contained 25 µg/ml of kanamycin and 25 µg/ml of chloramphenicol (final concentrations) at 37 °C with shaking at 160 rpm (overnight). Next, cultivated cells were transferred to 100 ml of fresh Luria-Bertani medium that contained 25 µg/ml of kanamycin and 25 µg/ml of chloramphenicol (final concentrations) and were cultivated for 2 h at 37 °C with shaking at 120 rpm. Isopropyl-$\beta$-D-thiogalactopyranoside (final concentration, 0.1 mM) was then added, and cultivation was continued for 18 h at 25 °C with shaking at 120 rpm. The cells were harvested by centrifugation (4,160 × *g*, 10 min, 4 °C), resuspended in 50 mM Tris–HCl buffer (pH 8.0), and then disrupted by sonication at 4 °C. Cellular debris was removed by centrifugation (20,000 × *g*, 30 min, 4 °C), and the supernatant was collected and purified with a Ni-affinity column, HisTrap HP (GE Healthcare). In addition, the active fraction was desalted with a PD-10 column (GE Healthcare) equilibrated with 50 mM Tris–HCl buffer (pH 8.0).

The L-amino acid ligase activity of recombinant RizA was assayed as follows unless otherwise specified: The standard reaction mixture (total volume, 0.2 ml) contained 0.1 mg/ml of purified His-tagged RizA, 12.5 mM ATP, 12.5 mM $MgSO_4 \cdot 7H_2O$, and 12.5 mM substrate(s) in 50 mM Tris–HCl buffer (pH 8.0). The reaction was performed at 30 °C for 18 h. To detect peptide synthesis activity, the amount of phosphate released by the reaction was determined with a Determiner L IP kit according to the manufacturer's protocol (Kyowa Medex, Tokyo). To confirm peptide synthesis in detail, reaction mixtures were analyzed by MALDI-TOFMS, LC-ESI-MS, HPLC, or NMR. When NMR analysis was performed, 50 mM potassium phosphate buffer (pH 8.0) was used for the reaction.

To investigate substrate specificity, every combination of one or two amino acids selected from the following amino acids was examined: L-arginine (Arg), L-lysine (Lys), L-histidine (His), L-glutamine (Gln), L-asparagine (Asn), L-glutamate (Glu), L-aspartate (Asp), L-alanine (Ala), L-serine (Ser), L-threonine (Thr), glycine (Gly), L-proline (Pro), L-valine (Val), L-leucine (Leu), L-isoleucine (Ile), L-methionine (Met), L-cysteine (Cys), L-phenylalanine (Phe), L-tryptophan (Trp), and L-tyrosine (Tyr). DL-2-Amino-5-phosphonopentanoic acid (APV) was used as a structural analog of L-2-amino-5-phosphono-3-*cis*-pentenoic acid (APPA), and a reaction with Arg plus APV was conducted. To test reactivity with D-amino acids, reactions with

**Table 1.** Primers

| Name | PCR template[a] | Primer (5′-3′) | Source |
|---|---|---|---|
| C1 | *Hin*dIII fragment and *Pst*I fragment | GTACATATTGTCGTTAGAACGCGTAATACGACTCA | Takara Bio |
| P1[b] | *Hin*dIII fragment | CTAGTCTAGAGATAAACCGGAACCGATTCAATTTTTTCAAAAAGA | This work |
| P2[b] | *Hin*dIII fragment | CTAGGGTACCATAGCACGATCTCGTAATGA | This work |
| P3[b] | *Pst*I fragment | CTAGGGATCCCCGTATATAGAGATCCAAAT | This work |
| V1[c] | Each PCR fragment | GTTTTCCCAGTCACGAC | This work |
| V2[c] | Each PCR fragment | CAATTAATGTGAGTTAG | This work |
| S1 | C1–P1 PCR fragment | ATAGCACGATCTCGTAATGA | This work |
| S2 | C1–P1 PCR fragment | CAATGAAGACTAAACTTAAA | This work |
| S3 | C1–P2 PCR fragment | TGAATTTCTAAAGGCATGTG | This work |
| S4 | C1–P2 PCR fragment | AACTCCTTTTCAATACAATA | This work |
| S5 | C1–P3 PCR fragment | CCGGATCATTTTGACTCAAA | This work |
| S6 | C1–P3 PCR fragment | CCGTATATAGAGATCCAAAT | This work |
| R1 | *B. subtilis* genome | GGTATTGAGGGTCGCATGCTTCGTATTTTA | This work |
| R2 | *B. subtilis* genome | AGAGGAGAGTTAGAGCCTTATTGGTTCATCAT | This work |

[a]*Hin*dIII fragment and *Pst*I fragment: Genomic DNA of *B. subtilis* NBRC3134 was digested with *Hin*dIII and with *Pst*I, and the resulting DNA fragments were ligated to the *Hin*dIII or to the *Pst*I cassette. PCR fragments were ligated into pUC19 vector, and the resulting plasmids were used to analyze DNA sequences as templates.
[b]P1 was designed on the basis of the N-terminal amino acid sequence of the purified enzyme. P2 and P3 were designed on the basis of sequence analysis of the C1–P1 PCR fragment.
[c]V1 and V2 were designed on the basis of the DNA sequence derived from pUC19 vector.

D-alanine (D-Ala) plus Arg, D-arginine (D-Arg) plus Ala, and D-Ala plus D-Arg were conducted. To characterize RizA, ATP was replaced with GTP, CTP, or TTP, and $MgSO_4 \cdot 7H_2O$ was replaced with $MnSO_4 \cdot 5H_2O$, $CoSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, or $CaSO_4 \cdot 2H_2O$. To determine the optimum pH, 50 mM $NaHCO_3$ buffer (pH 9.0 to 11.0) and 50 mM GTA buffer (50 mM 3,3-dimethylglutarate, 50 mM Tris, and 50 mM 2-amino-2-methyl-1,3-propanediol, pH 7.0 to 10.0) were used, and the reaction was performed at 37 °C for 1 h. Arg (25 mM) was used as substrate, and the amount of L-arginyl-L-arginine (Arg-Arg) was measured by HPLC. To determine the optimum temperature, the reaction temperature was varied between 20 °C and 60 °C. Arg (25 mM) was used as substrate, and the reaction was performed in 50 mM GTA buffer (pH 9.5) for 1 h. The amount of Arg-Arg was measured by HPLC.

*Analysis.*

*(i) MALDI-TOFMS.* The reaction mixture was diluted 50-fold with Milli-Q water. The diluted sample (2 µl) was mixed with the matrix solution (2 µl) on a plate. The matrix (2,5-dihydroxybenzoic acid, DHB, 10 mg) was dissolved in the solvent (acetonitrile:0.1% trifluoroacetic acid = 2:3, 1 ml). The plate was dried and loaded into MLADI-TOFMS (AXIMA-CFR, Shimadzu, Kyoto, Japan).

*(ii) LC-ESI-MS.* An aliquot (5 µl) of the reaction mixture was injected into the LC-ESI-MS system (HPLC, Agilent 1100 series, Agilent Technologies, Santa Clara, CA; ESI-MS, LCQ Deca, Thermo Scientific, Waltham, MA). A Sunfire $C_{18}$ column (5 µm, Waters, Milford, MA) was used. Formic acid solution (50 mM) and acetonitrile were prepared as solvent A and solvent B respectively, and the column was equilibrated with solvent A. The analysis was performed with the following program: 0 to 2 min, held at A:B of 100:0; 2 to 9 min, a linear increase to A:B of 0:100; 9 to 15 min, held at A:B of 0:100; 15 to 20 min, held at A:B of 100:0. The flow rate was 1 ml/min, and the column temperature was 35 °C. After column separation by HPLC, the reaction products were analyzed by ESI-MS.

*(iii) HPLC.* Amino acids were derivatized with N-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA). The sample mixture was analyzed with the HPLC system (L-2000 series, Hitachi High Technologies, Tokyo) using a WH-C18A column (3 µm, Hitachi High Technologies). The details of the analytical procedure are described in our previous report.[20]

*(iv) NMR.* $^1$H-NMR spectra and $^1$H–$^{13}$C heteronuclear multiple bond correlation (HMBC) NMR spectra were acquired with a DMX500 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany).[3] 3-Trimethylsilyl-tetradeuterosodium propionate (TSP) was added as an internal standard and chemical shift standard in $^1$H-NMR analysis. Dioxane in deuterium oxide was used as external standard and chemical shift standard in $^{13}$C-NMR analysis. Analysis was performed by Kyowa Hakko Kogyo (Tokyo).

# Results

*Confirmation of L-amino acid ligase activity using the cell-free extract of* B. subtilis *NBRC3134*

We formulated the hypothesis that an L-amino acid ligase plays a role in the formation of $\alpha$-peptide bonds in rhizocticin biosynthesis, and that *B. subtilis* NBRC3134 possesses the L-amino acid ligase that takes Arg as an N-terminal substrate, because all rhizocticins have Arg-APPA. We assumed that the reaction mechanism of L-amino acid ligase is basically the same as those of D-alanine-D-alanine ligase (EC 6.3.2.4), glutathione synthetase (EC 6.3.2.3), and glutamine synthetase (EC 6.3.2.1), because these enzymes including L-amino acid ligase all belong to the ATP-dependent carboxylate-amine/thiole ligase superfamily.[21,22] Therefore, L-amino acid ligase should take aminoacylphosphate as the reaction intermediate. Enzyme activities taking such reaction intermediates can be detected by colorimetric assay, reacting with an amino acid and $NH_2OH$ to form aminoacyl hydroxamate, which forms a red complex with Fe(III).[16] We confirmed that the YwfE protein synthesized L-alanine hydroxamate (data not shown), indicating that the method is effective to detect L-amino acid ligase activity. Then we examined Arg-NHOH synthesis using cell-free extracts of *B. subtilis* NBRC3134. As expected, Arg-NHOH was detected in the reaction mixture by the colorimetric method, and was additionally detected by HPLC. This result suggests that the enzyme catalyzing Arg-specific $\alpha$-peptide bond formation existed in the cell-free extract of *B. subtilis* NBRC3134.

*Enzyme purification from* B. subtilis *NBRC3134*

Enzyme purification was carried out by detecting Arg-NHOH synthesis activity, as described in "Materials and Methods." The purification steps are summarized in Table 2. The enzyme was finally purified 1,280-fold with 0.8% yield, and its specific activity was 2.02 U/mg. The major protein band of the purified enzyme on SDS–PAGE gel gave an estimated molecular mass of approximately 45 kDa (Fig. 1). Furthermore, the native molecular mass of the enzyme was estimated to be

**Table 2.** Purification of L-Amino Acid Ligase from *B. subtilis* NBRC3134

| Step | Total protein (mg) | Total activity (U[a]) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Cell-free extract | 415 | 0.655 | 0.00158 | 1.00 | 100 |
| $(NH_4)_2SO_4$ precipitation | 7.85 | 0.176 | 0.0224 | 14.2 | 26.8 |
| Butyl FF | 0.727 | 0.0587 | 0.0808 | 51.1 | 8.96 |
| Mono Q | 0.00933 | 0.00882 | 0.946 | 598 | 1.35 |
| Superdex 200 | 0.00257 | 0.00520 | 2.02 | 1280 | 0.793 |

[a]One unit (1 U) of enzyme activity was defined as the amount of enzyme that produces 1 µmol of Arg-NHOH per min.

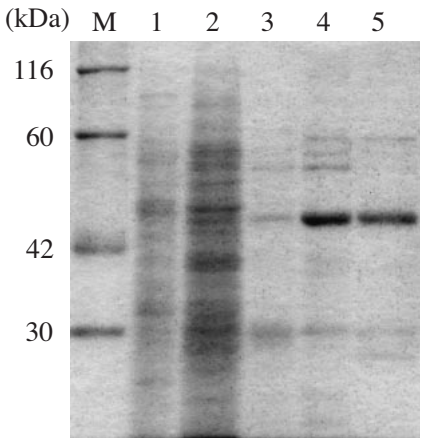


**Fig. 1.** SDS–PAGE Analysis of the Purified L-Amino Acid Ligase from *B. subtilis* NBRC3134.

Lanes: M, molecular mass standard; 1, cell-free extract of *B. subtilis* NBRC3134; 2, $(NH_4)_2SO_4$ precipitation; 3, HiPrep 16/10 Butyl FF column; 4, Mono Q 5/50 GL column; and 5, HiLoad 16/60 Superdex 200 pg column.

80 kDa by gel-filtration chromatography. Thus the purified enzyme might have been a homo-dimer.

*Cloning of the gene encoding the purified enzyme*

The N-terminal amino acid sequence of the purified enzyme was determined to be MLRILLINSDKPE-PIQFFQKDKETN. A BLAST search was performed using the N-terminal amino acid sequence as a query, but no homologous proteins were detected. The cassette PCR method was used to obtain a whole DNA sequence of the gene encoding the purified enzyme.[19] A P1 primer was designed on the basis of the N-terminal amino acid sequence (Table 1). Southern hybridization analysis showed that a 2.5-kbp DNA fragment produced by digestion of genomic DNA with *Hin*dIII and an 8.0-kbp DNA fragment produced by digestion of genomic DNA with *Pst*I contained the N-terminal region of the purified enzyme (data not shown). PCR amplification and sequence analysis were performed, and an ORF of 1,242 bp was obtained (DDBJ accession no. AB437349). This ORF coded for 413 amino acid residues with a molecular weight of 46,344. The molecular size and the N-terminal amino acid sequence of the protein encoded by this ORF were identical to those of the purified enzyme from *B. subtilis* NBRC3134. Furthermore, a Pfam database search indicated that this protein had an ATP-grasp motif (PF02222).[23] The gene encoding the purified enzyme was designated *rizA*.

*Characterization of recombinant RizA*

RizA was produced by the *E. coli* gene-expression system as an N-terminal His-tagged protein, and was purified to homogeneity with a Ni-affinity column. To test the substrate specificity, every combination of 20 amino acids (400 kinds of reactions in total) was examined under standard reaction conditions. First, the amount of released phosphate in the reaction mixture was measured. Measurement of released phosphate provides information on the reaction because ATP is hydrolyzed to ADP and phosphate when L-amino acid ligase synthesizes dipeptide. A reaction in the absence of amino acid as substrate was additionally conducted as a blank test, and all the data were subtracted from this blank value (1.2 mM). Phosphate analysis showed that 2 to 4 mM phosphate was released in the 20 reaction mixtures that contained Arg as a substrate, but that little phosphate was released in the reaction mixtures that did not contain Arg. Next, 19 reaction mixtures that contained Arg and Xaa (where Xaa is an arbitrary amino acid) as substrates were analyzed by MALDI-TOFMS and LC-ESI-MS. This analysis showed that 18 hetero-dipeptides consisting of Arg and Xaa were synthesized, but the hetero-dipeptide consisting of Arg and Pro was not detected. In addition, no tripeptides and no longer peptides were detected. A LC-ESI-MS chart of the reaction using Arg and His as substrates is shown in Fig. 2. Finally, NMR analysis showed that RizA synthesized hetero-dipeptide and that Arg was present at its N-terminus (Fig. 3). RizA synthesized Arg-Arg when only Arg was used as substrate, but preferentially synthesized Arg-Xaa when Arg and other amino acids were used as substrates (Table 3). Arg and APV, structural analog of APPA, were additionally conducted (Supplemental Fig. 2; see *Biosci. Biotechnol. Biochem.* Web site), and NMR analysis showed that RizA synthesized Arg-APV (Supplemental Fig. 3; see *Biosci. Biotechnol. Biochem.* Web site). Furthermore, RizA did not use D-form amino acids as substrates.

ATP and $Mg^{2+}$ were essential for L-amino acid ligase activity, and ATP was hydrolyzed to ADP and phosphate. In addition, ATP was not replaced by GTP, CTP, or TTP. $Mg^{2+}$ was replaced by $Mn^{2+}$ and $Co^{2+}$, but the use of $Mn^{2+}$ and of $Co^{2+}$ decreased L-amino acid ligase activity.

The effects of pH and temperature on L-amino acid ligase activity were examined (Fig. 4). The optimum pH was around 9.5, and the activity decreased sharply below 9.0 and above 10.0. The optimum temperature was around 37 °C, and a significant decrease was observed above 50 °C.

## Discussion

We obtained a novel L-amino acid ligase RizA from *B. subtilis* NBRC3134 producing peptide-antibiotic rhizocticin by enzyme purification, although several

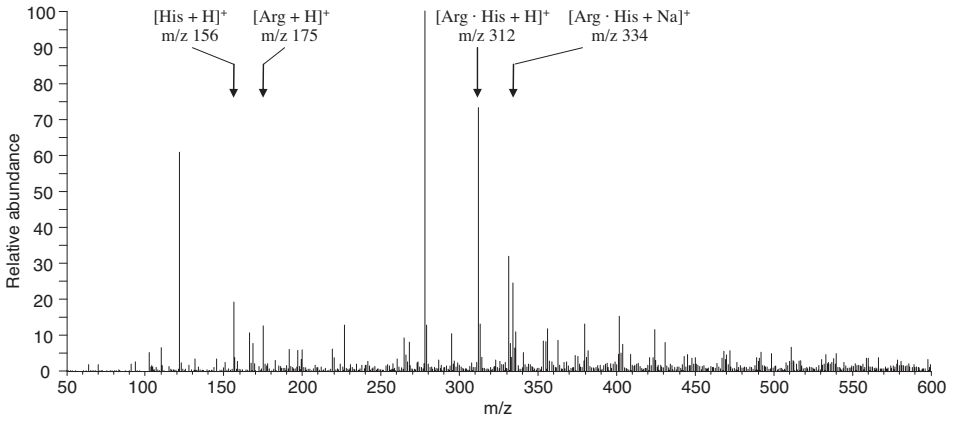


**Fig. 2.** LC-ESI-MS Analysis of the Reaction Catalyzed by RizA.
Arg and His were used as substrates. Peaks assigned are as follows: m/z 156, $[His + H]^+$; m/z 175, $[Arg + H]^+$; m/z 312, $[Arg{\cdot}His + H]^+$; m/z 334, $[Arg{\cdot}His + Na]^+$. [Arg·His] means [Arg-His] or [His-Arg].

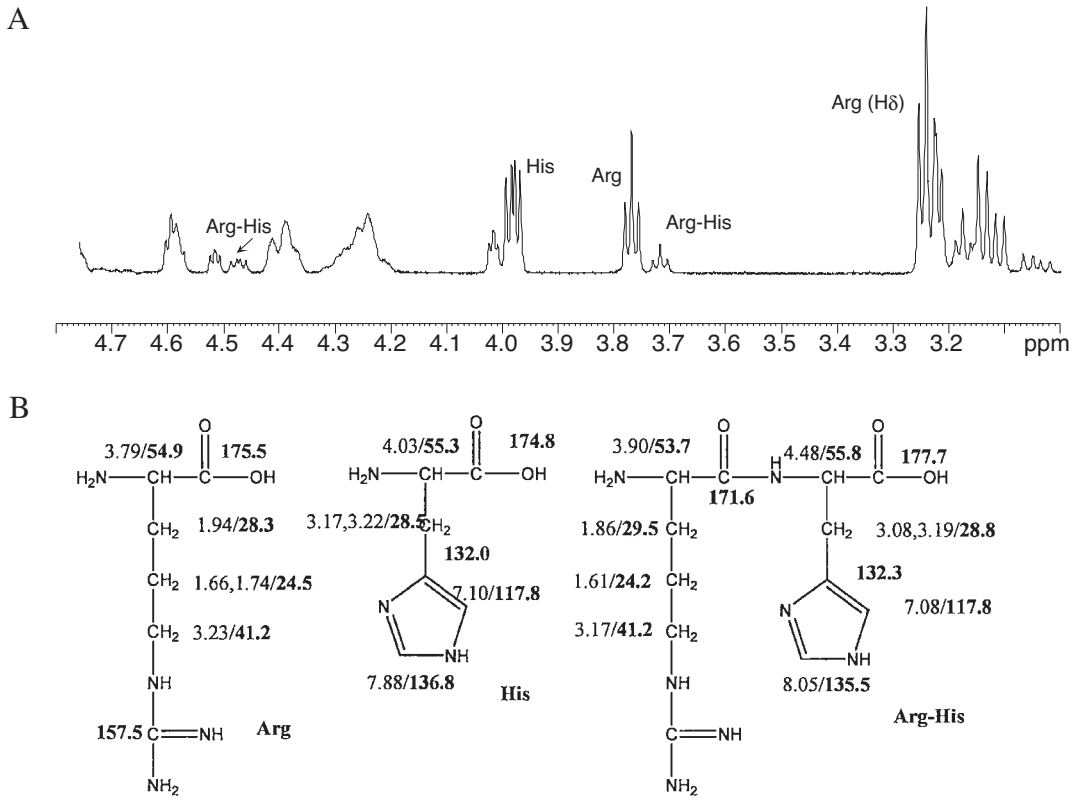


**Fig. 3.** NMR Analysis of the Reaction Mixture in Which Arg and His Were Used as the Substrates.
$^1$H-NMR (A) and FG-HMBC (B) analyses were performed. Figure B: normal font, chemical shift of $^1$H; bold font, chemical shift of $^{13}$C.

**Table 3.** Structural Determination and Quantitative Analysis of Dipeptides Synthesized by RizA

| Substrate 1 | Substrate 2 (Xaa) | Product[a] (mM) | | | |
|---|---|---|---|---|---|
| | | Arg-Arg | Xaa-Xaa | Arg-Xaa | Xaa-Arg |
| Arg | Arg | 1.7 | — | — | — |
| | Ala | n.d. | n.d. | 0.9 | n.d. |
| | Ser | n.d. | n.d. | 3.1 | n.d. |
| | His | n.d. | n.d. | 1.9 | n.d. |

[a]n.d., not detected.

L-amino acid ligases were obtained by *in silico* screening.[1,3,4] This is the first report on the purification of native-form L-amino acid ligase. We used the colorimetric method, detecting aminoacyl hydroxamate, to detect L-amino acid ligase activity for the first time, although this method itself has been reported[16] and glutamine synthetase activity is often assayed by it.[24,25] Thus we offer a new way to find an L-amino acid ligase. Our method has several advantages: (i) it is an easy procedure; (ii) enzyme activity is checked visually; and (iii) a synthesized hydroxamate is not easily decomposed by cellular peptidases. If amino acids are used as substrates to detect L-amino acid ligase activity, the synthesized peptide is decomposed rapidly by cellular peptidases, and as a result, L-amino acid ligase activity is not detected easily and purification is not successful.

We cloned and overexpressed *rizA* in *E. coli* cells,

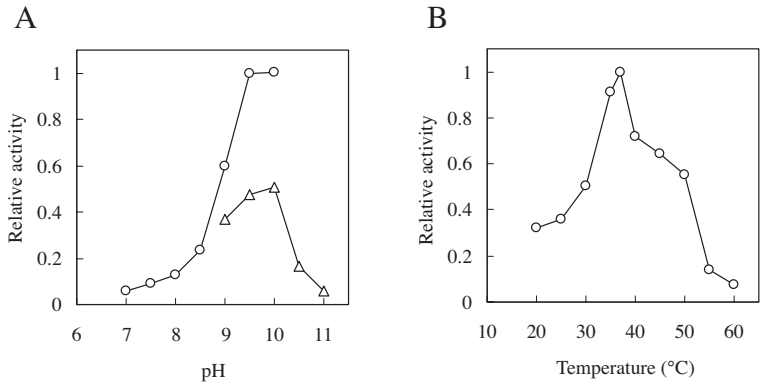


**Fig. 4.** Effects of (A) pH and (B) Temperature on the L-Amino Acid Ligase Activity of Recombinant RizA.
The reaction was performed as described in "Materials and Methods." The activity was assayed by measuring the amount of Arg-Arg synthesized in the reaction mixture. In Figure A, circles indicate 50 mM GTA buffer (pH 7.0 to 10.0), and triangles indicate 50 mM $NaHCO_3$ buffer (pH 9.5 to 11.0).

and found that recombinant RizA synthesized Arg-Xaa dipeptides in an ATP-dependent manner. MS and NMR analysis showed that RizA had strict substrate specificity toward Arg as the N-terminal substrate; on the other hand, the substrate specificity at the C-terminus was very relaxed. RizA has novel substrate specificity and is the first L-amino acid ligase which takes a positively charged amino acid as the N-terminal substrate. A Pfam database search indicated that RizA belongs to the ATP-dependent carboxylate-amine/thiol ligase superfamily, like already-known L-amino acid ligases. However, the amino acid sequence of RizA had low homology with them and had 21% identity to that of the YwfE protein (DDBJ accession no. AL009126), 20% identity to that of the RSp1486a protein (DDBJ accession no. AB428352), and 20% identity to that of the BL00235 protein (DDBJ accession no. CP000002).[1,3,4] Furthermore, RizA is a homo-dimer although the RSp1486a and BL00235 proteins were monomer enzymes. The optimum pH and temperature were 9.5 and 37 °C, similar to those of already-known L-amino acid ligases.

At the beginning of this study, we formulated the hypothesis that an L-amino acid ligase plays a role in the formation of $\alpha$-peptide bonds in rhizocticin biosynthesis. We could not confirm that RizA synthesized rhizocticin A (Arg-APPA) due to the unavailability of APPA. Taking into account the substrate specificity of RizA, we expect that RizA is one of the enzymes involved in rhizocticin biosynthesis. In addition, no genes encoding proteins homologous to RizA were identified in the genome of *B. subtilis* 168 (DDBJ accession no. AL009126), which does not produce rhizocticins. Based on reports that the genes involved in antibiotic biosynthesis form a gene cluster,[1,26,27] we concluded that the genes involved in rhizocticin biosynthesis might form a cluster. Hence DNA sequence analysis around *rizA* is now under investigation. We have identified genes that show similarity to *ppm* (the phosphoenolpyruvate phosphomutase gene, GenBank accession no. AY632421) and *ppd* (the phosphonopyruvate decarboxylase gene, GenBank accession no. AY632421), which are involved in phosphinothricin biosynthesis, from *Streptomyces viridochromogenes* DSM40736.[28] It has been reported that phosphomutase and decarboxylase are involved in the synthesis of C-P compounds such as phosphinothricin and fosfomycin.[29] APPA is also a C-P compound, and the genes that we identified might be involved in APPA biosynthesis. We also expect that other L-amino acid ligase might catalyze tripeptide synthesis and ligate Val, Ile, or Leu to Arg-APPA in rhizocticin B, C, or D biosynthesis, because RizA had no tripeptide synthesis activity. Our research might lead to the discovery of a novel L-amino acid ligase that catalyzes tripeptide synthesis and to clarification of the mechanism of rhizocticin biosynthesis.

In conclusion, we obtained a novel L-amino acid ligase RizA from *B. subtilis* NBRC3134, and it synthesized Arg-Xaa dipeptides. RizA had novel substrate specificity, and the use of RizA in dipeptide production should extend the variety of dipeptides that are synthesized using L-amino acid ligase.

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