

Discovery of novel L-amino acid α -ligases and application to dipeptide synthesis.

Toshinobu Arai¹, Yuji Nakazawa¹, Atsushi Noguchi¹, Makoto Yagasaki², Kohtaro Kirimura¹, and Kuniki Kino^{1*}

¹Department of Applied Chemistry, School of Science and Engineering, Waseda University, Ohkubo Shinjuku-ku Tokyo, 169-8555, Japan; ²Technical Research Laboratories, Kyowa Hakko Kogyo Co. Ltd, Hofu-shi Yamaguchi, 747-8522, Japan
E-mail: kkino@waseda.jp

Introduction

Dipeptides and their derivatives have good physical properties or various physiological functions. For example, Ala-Gln has improved physical stability and solubility in comparison to Gln, and also Val-Tyr has an antihypertensive effect. Organic synthesis and reverse reaction of protease were used as conventional methods for production of dipeptides. However, these methods were not effective with regard to yield and cost. Recently, a novel enzyme, L-amino acid α -ligase (Lal) was discovered. YwfE from *Bacillus subtilis* 168 was reported as a first enzyme of Lal [1]. This enzyme catalyzes the formation of an α -peptide bond from two L-amino acids in an ATP-dependent manner (Fig. 1). This reaction goes only one direction, and also there is no need for protecting groups on substrate amino acids. These points are of great advantage for efficient production of dipeptides. Actually, utilization of Lal has made it possible to develop an effective method for industrial dipeptide production. However, the utilization of Lal for dipeptide production was limited because of its own substrate specificity. In this study, we searched for novel Lals to enlarge the range of utilization of Lal for dipeptide production.

Results and Discussion

First, a BLAST search was performed using the amino acid sequence of YwfE as a query. Several hypothetical proteins were selected from the result (Table 1). Next, those proteins were produced with an *Escherichia coli* gene-expression system as a C-terminal 6 His-tagged protein. Then, Lal activity was assayed by the detection of released phosphate that was caused by ATP-hydrolysis. Hypothetical proteins; RSp1486a from *Ralstonia solanacearum* JCM 10489 and BL00235 from *Bacillus licheniformis* NBRC 12200, showed significant amount of released phosphate. Additionally, reaction mixtures were analyzed by MALDI-TOF-MS and NMR. Summary of substrate specificities is shown in Fig. 2. It was found that RSp1486a had broad substrate specificity. On the other hand, BL00235 showed narrow substrate specificity, and Met and Leu were arranged at the N-terminus of the dipeptide. These proteins had clearly different substrate specificities from YwfE. Next, the results of quantitative analysis are shown in Tables 2 and 3. When two different amino acids were used as substrates, RSp1486a preferentially synthesized hetero-dipeptide whose N-terminus was constituted of a more bulky amino acid. This property was opposite to that of YwfE which preferentially synthesized hetero-dipeptide whose N-terminus consisted of small size amino acids. And also, BL00235 synthesized only hetero-dipeptide, and homo-dipeptide was not synthesized at all.

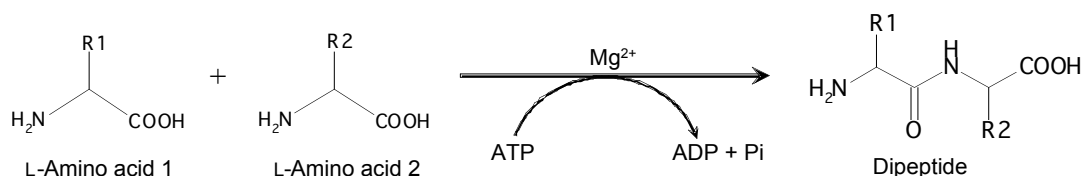


Fig. 1. Dipeptide synthesis catalyzed by Lal.

Table 1. Candidates for novel Lal.

| Name | Microorganism | AA | Homology (%) |
|----------|--|-----|--------------|
| YwfE | <i>Bacillus subtilis</i> 168 | 472 | - |
| RSp1486a | <i>Ralstonia solanacearum</i> JCM 10489 | 449 | 27.0 |
| SCO6437 | <i>Streptomyces coelicolor</i> A3(2) | 424 | 25.4 |
| BC3196 | <i>Bacillus cereus</i> ATCC 14579 | 423 | 24.5 |
| mlr6296 | <i>Mesorhizobium loti</i> MAFF 303099 | 407 | 24.1 |
| BCE3245 | <i>Bacillus cereus</i> ATCC 10987 | 419 | 24.0 |
| BL00235 | <i>Bacillus licheniformis</i> NBRC 12200 | 425 | 23.6 |

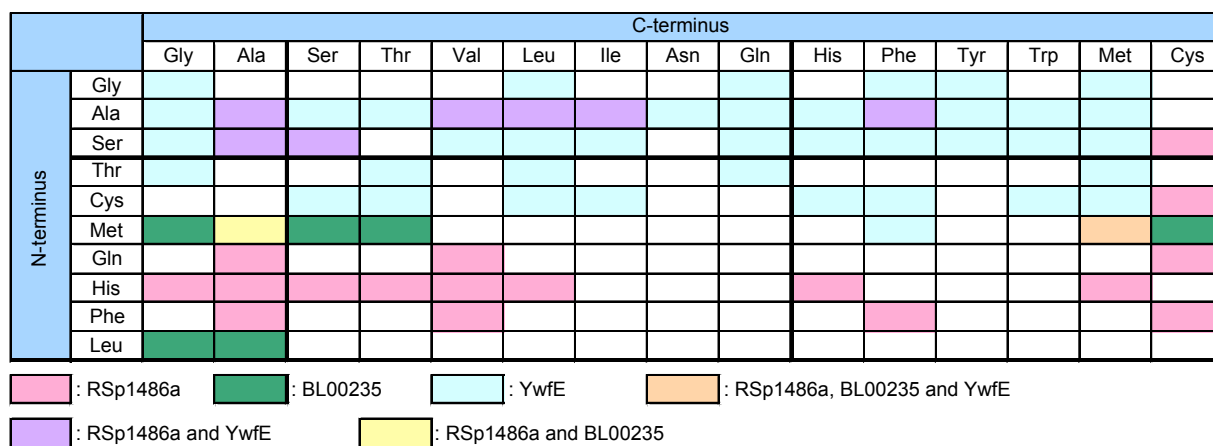


Fig. 2. Summary of substrate specificities.

Table 2. Quantitative analysis of reaction mixture of RSp1486a.

| Substrate | Product | | | | | | | | |
|-----------|---------------------|---------|-----|---------|-----------------------|---------|------|---------|------|
| | Homo-Dipeptide (mM) | | | | Hetero-Dipeptide (mM) | | | | |
| Ala | Ala-Ala | 10.1 | | | | | | | |
| Phe | Phe-Phe | 8.3 | | | | | | | |
| Ala | Phe | Ala-Ala | 2.0 | Phe-Phe | 0.9 | Ala-Phe | N.D. | Phe-Ala | 8.7 |
| Ala | His | Ala-Ala | 1.2 | His-His | N.D. | Ala-His | N.D. | His-Ala | 10.9 |
| Ala | Gln | Ala-Ala | 2.5 | Gln-Gln | N.D. | Ala-Gln | N.D. | Gln-Ala | 8.9 |
| Ala | Ser | Ala-Ala | 2.6 | Ser-Ser | N.D. | Ala-Ser | N.D. | Ser-Ala | 6.5 |
| His | Ser | His-His | 0.8 | Ser-Ser | N.D. | Ser-His | N.D. | His-Ser | 6.2 |

N.D.: Not detected. Reaction mixture contained 12.5 mM L-Amino acid 1, 12.5 mM L-Amino acid 2, 12.5 mM ATP, 12.5 mM MgSO₄, and 0.065 mg/mL purified enzyme in 50 mM sodium phosphate buffer pH 8.0. Reaction was performed at 30°C for 20 hr.

Table 3. Quantitative analysis of reaction mixture of BL00235.

| Substrate | Product | | | | | | | | |
|-----------|---------------------|---------|------|---------|-----------------------|---------|------|---------|-----|
| | Homo-Dipeptide (mM) | | | | Hetero-Dipeptide (mM) | | | | |
| Met | Ala | Met-Met | N.D. | Ala-Ala | N.D. | Ala-Met | N.D. | Met-Ala | 5.5 |
| Met | Cys | Met-Met | N.D. | Cys-Cys | N.D. | Cys-Met | N.D. | Met-Cys | 2.6 |
| Leu | Ala | Leu-Leu | N.D. | Ala-Ala | N.D. | Ala-Leu | N.D. | Leu-Ala | 2.2 |

N.D.: Not detected. Reaction condition was the same as that of RSp1486a except that 0.5 mg/mL purified enzyme was used.

Furthermore, as a part of the novel Lal search, homologous enzymes were sought from other *Ralstonia* species. First, the search for homologous enzymes from other *Ralstonia solanacearum* was performed with the PCR technique (Table 4). PCR primers were designed from DNA sequence of RSp1486a. From all eleven *R. solanacearum* we examined, homologous enzymes were obtained. Their homologies of amino acid sequence were very high (93-95%). The result of released phosphate analysis, when homo-dipeptide synthesis was examined, is shown in Fig. 3. All these homologous enzymes showed the same substrate specificities. In addition, when hetero-dipeptide synthesis was examined, they showed the same substrate specificities (data not shown). It was suggested that *Ralstonia solanacearum* generally had the Lal gene, and these enzymes had same substrate specificities.

Table 4. List of other *Ralstonia solanacearum* for homologous enzyme search.

| Name | Microorganism | |
|----------|-------------------------------|-------------|
| RSp1486a | <i>Ralstonia solanacearum</i> | JCM 10489 |
| RS-1 | <i>Ralstonia solanacearum</i> | MAFF 211270 |
| RS-2 | <i>Ralstonia solanacearum</i> | MAFF 211272 |
| RS-3 | <i>Ralstonia solanacearum</i> | MAFF 211282 |
| RS-4 | <i>Ralstonia solanacearum</i> | MAFF 211396 |
| RS-5 | <i>Ralstonia solanacearum</i> | MAFF 211403 |
| RS-6 | <i>Ralstonia solanacearum</i> | MAFF 211544 |
| RS-7 | <i>Ralstonia solanacearum</i> | MAFF 301520 |
| RS-8 | <i>Ralstonia solanacearum</i> | MAFF 301522 |
| RS-9 | <i>Ralstonia solanacearum</i> | MAFF 301523 |
| RS-10 | <i>Ralstonia solanacearum</i> | MAFF 301526 |
| RS-11 | <i>Ralstonia solanacearum</i> | MAFF 301560 |

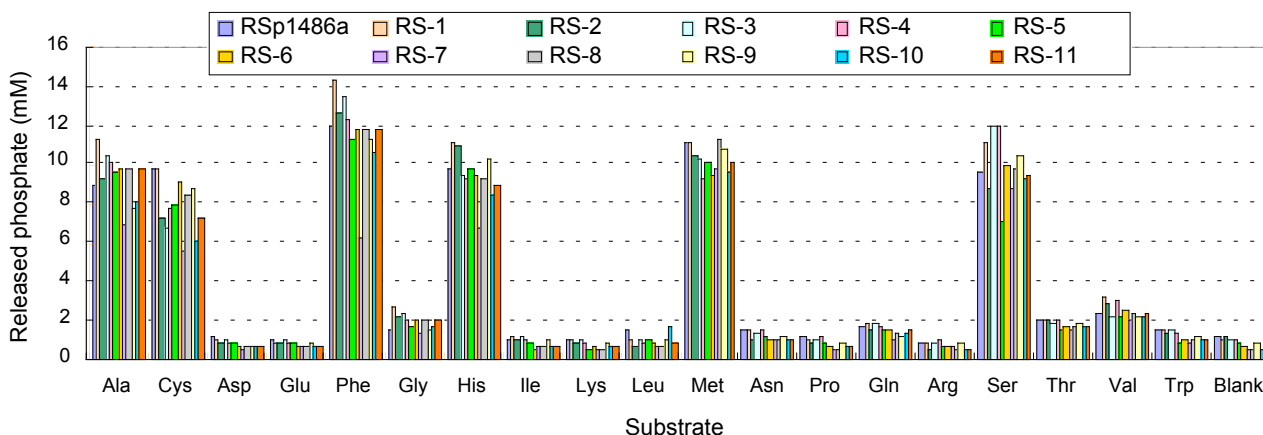


Fig. 3. Lal activity of homologous enzyme from other *R. solanacearum* (homodipeptide production).

Next, the search of homologous enzyme from other *Ralstonia* species was performed with southern hybridization (Table 5.). RSp1496a gene was used as a template to synthesize DIG-labeled probe (random priming method). The result of southern hybridization is shown in Fig. 4. At lane No.10, DNA fragment of Rsp1486a was hybridized as a positive control. However, at other lanes, hybridization was not detected. This result suggested that there was no homologous enzyme in other *Ralstonia* species we examined.

Table 5. List of other *Ralstonia* species for homologous enzyme search.

| No. | Microorganism |
|-----|--|
| 1 | <i>Ralstonia oxalaticus</i> NBRC 13593 |
| 2 | <i>Ralstonia eutropha</i> IAM 12368 |
| 3 | <i>Ralstonia pickettii</i> IAM 13530 |
| 4 | <i>Ralstonia metalophila</i> IAM 14785 |
| 5 | <i>Ralstonia silverii</i> IAM 14786 |
| 6 | <i>Ralstonia tsushima</i> IAM 14787 |
| 7 | <i>Ralstonia gilardii</i> JCM 11283 |
| 8 | <i>Ralstonia mannitolilytica</i> JCM 11284 |
| 9 | <i>Ralstonia paucula</i> JCM 11286 |
| 10 | <i>Ralstonia solanacearum</i> JCM 10489 |

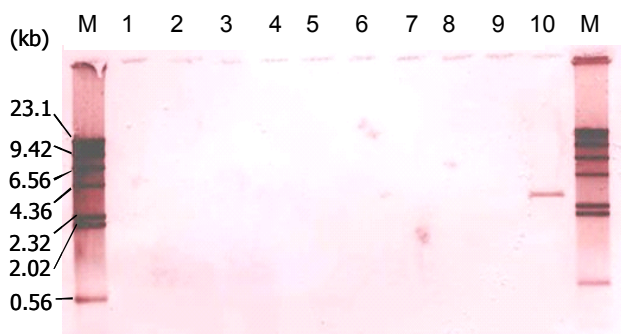


Fig. 4. Result of southern hybridization analysis.

* Lane No. in Fig. 4 corresponded to No. in Table 5.

We discovered novel two Lals, RSp1486a and BL00235, by *in silico* analysis. These enzymes had clearly different substrate specificities from YwfE. They will contribute to enlarge the range of utilization of Lal for dipeptide production and facilitate effective dipeptide production methods.

Acknowledgments

We thank Ritsuko Katahira (Kyowa Hakko Kogyo Co. Ltd.) for skillful ¹H-NMR analysis. This work is done at the “Center for Practical Chemical Wisdom” supported by Global COE program of MEXT.

References

1. Tabata K, Ikeda H, Hashimoto S. (2005) *J Bacteriol.*, **187**, 5195-202.