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Effective production of Pro–Gly by mutagenesis of L-amino acid ligase

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L-Amino acid ligase (Lal) catalyzes dipeptide synthesis from unprotected L-amino acids by hydrolysis ATP to ADP. Each Lal displays unique substrate specificity, and many different dipeptides can be synthesized by selecting suitable Lal. We have already successfully synthesized Met–Gly selectively by replacing the Pro85 residues of Lal from *Bacillus licheniformis* (BL00235). From these results, we deduced that the amino acid residue at position 85 had a key role in enzyme activity, and applied these findings to other Lals. When Pro and Gly were used as substrates, TabS from *Pseudomonas syringae*, synthesized the salt taste enhancing dipeptide Pro–Gly and other three dipeptides (Gly–Pro, Pro–Pro, and Gly–Gly) was hardly synthesized from its substrate specificity. However, the amount of Pro–Gly was low. Therefore, to alter the substrate specificity and increase the amount of Pro–Gly, we selected amino acid residues that might affect the enzyme activity, Ser85 corresponding to Pro85 of BL00235, and His294 on the results from previous studies and the predicted structure of TabS. These residues were replaced with 20 proteogenic amino acids, and Pro–Gly synthesizing reactions were conducted. The S85T and the H294D mutants synthesized more Pro–Gly than wild-type. Furthermore, the S85T/H294D double mutant synthesized considerably more Pro–Gly than the single mutant did. These results showed that the amino acid position 85 of TabS affect the enzyme activity similarly to BL00235. In addition, replacing the amino acid residue positioning around the N-terminal substrate and constructing the double mutant led to increase the amount of Pro–Gly.

[**Key words:** Dipeptide; L-Amino acid ligase; Site-directed mutagenesis; TabS; Salt taste enhancer]

L-Amino acid ligase (Lal) belongs to a member of the ATP-dependent carboxylate-amine/thiol ligase superfamily (1) and catalyzes dipeptide synthesis from unprotected L-amino acids by hydrolysis of ATP to ADP (2). The first report of Lal was YwfE from *Bacillus subtilis* 168 in 2005 (2), and about 20 different Lals have subsequently been discovered. Each Lal characteristically has a unique substrate specificity. For instance, RizA from *B. subtilis* NBRC3134 accepts only Arg for the N-terminal substrate (3). BL00235 from *Bacillus licheniformis* NBRC12200 also shows strict substrate specificity, and accepts only Met and Leu as the N-terminal substrates (4). However, TabS from *Pseudomonas syringae* NBRC14081 show broad substrate specificity, and dipeptides are detected in 136 of 231 reaction mixtures containing one or two amino acids that are selected from 20 proteogenic amino acids and β-Ala as substrates (5). In previous research, we synthesized many dipeptides by selecting suitable Lals; however, the amount of synthesized dipeptide was sometimes small and some dipeptides were synthesized as by-products (3–5).

To solve these problems, we altered the substrate specificity of BL00235, and succeeded in obtaining the mutant that synthesized Met–Gly selectively as a salt taste enhancer by site-directed mutagenesis based on the crystal structure of BL00235 (PDB ID: 3VOT) (6–8). To our knowledge, this was the first report of the successful synthesis of useful dipeptide selectively by using Lal for site-directed mutagenesis. We focused on the residue determining the C-terminal substrate and constructed the mutant that synthesized Met–Gly selectively by replacing Pro85 residue with Phe or Tyr. Shomura et al. also reported that Leu110 residue of YwfE, which correspond to Pro85 residue of BL00235, is one of the residues which surrounding the aromatic ring of C-terminal amino acid substrate based on its structure (PDB ID: 3VMM) (9). We deduced that the amino acid residue at position 85 had a key role in the enzyme activity from these reports. Therefore, we applied these findings to other Lals.

In the present study, we focused on Pro–Gly which is a salt taste enhancing dipeptide (Kino, H. et al., Japanese patent application JP2015-128323, 2015) and a therapeutic agent (10). When 20 mM Pro and 20 mM Gly were used as substrate, TabS synthesized 8.3 mM Pro–Gly. Furthermore, TabS hardly synthesized few other three dipeptides, Pro–Pro, Gly–Pro and Gly–Gly, from its substrate specificity of preferring Pro and Gly as the N-terminal substrate and the C-terminal substrate, respectively (5). It would be possible to increase the amount of Pro–Gly by replacing the suitable amino acid residues of TabS with other amino acid residues. We selected the amino acid residues, which might affect the enzyme activity, based on the information described above and the predicted structure of TabS. Alignment of amino acid sequences of five Lals (2–5,11) are shown in Fig. 1, and predicted structure of TabS is shown in Supplemental Fig. S1. We focused on Ser85 residue of TabS which corresponds to Pro85 residue of BL00235 and

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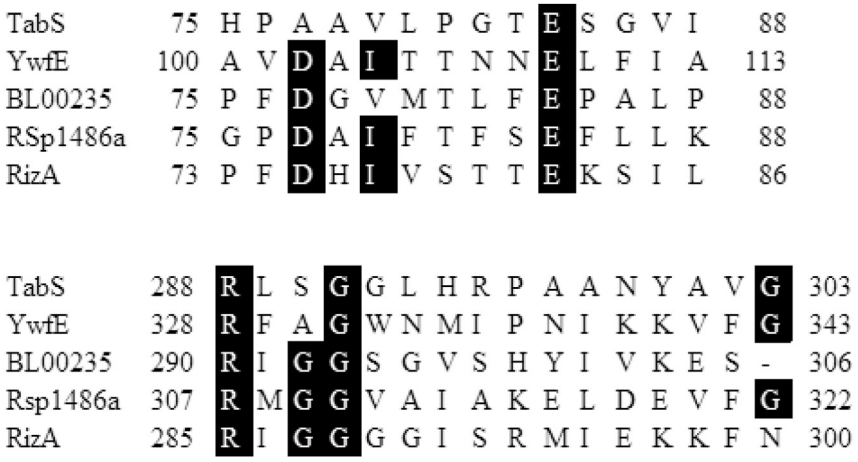

FIG. 1. Alignment of primary structures of Lals. Amino acid conserved among the five sequences of Lals, TabS (GenBank BAJ15424.1 from *P. syringae*), YwfE (CAB15798.1 from *B. subtilis*, BL00235 AAU25674.1 from *B. licheniformis*), Rsp1486a (BAG31900.1 from *R. solanacearum*), and RizA (BAG72134.1 from *B. subtilis*), in white with black shading.

Leu110 residue of YwfE and positions around C-terminal substrate (Fig. 1). Furthermore, we focused on the His294 residue which positions around N-terminal substrate (Fig. S1). This residue corresponds to Met334 residue of YwfE, which is a one of residues for determining the N-terminal substrate specificity (12). In the present study, Ser85 and His294 residues were replaced with 20 proteogenic amino acids, and Pro–Gly-synthesizing reactions were conducted. We described the mutants that synthesized more Pro–Gly than the wild-type TabS, and the characteristics of the mutants in detail.

MATERIALS AND METHODS

Materials All chemicals used in this study are commercially available and are of a chemically pure grade.

Site-directed mutagenesis Mutations were introduced by polymerase chain reaction (PCR) with KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) based on the method on a QuikChange site-directed mutagenesis kit (Agilent Technologies, CA, USA) using oligonucleotide primers. The generation of the desired mutations was confirmed through DNA sequencing.

Enzyme preparation The genes encoding TabS were previously cloned into the pET28a(+) vectors (5). Recombinant *Escherichia coli* BL21(DE3) cells were precultivated in 3 mL LB medium (1% bacto tryptone, 0.5% yeast extract, 1% NaCl) with 30 μg/mL kanamycin at 37°C for 5 h with shaking at 160 rpm. For the main culture, 200 mL LB medium containing 30 μg/mL kanamycin was inoculated with 3 mL preculture broth and cultivated with shaking at 37°C at 120 rpm. After cultivating for 1 h, 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to the medium, and cultivation conducted at 25°C for 19 h with shaking at 120 rpm. The cells were collected with centrifugation (3000 ×*g*, 10 min, 4°C) and washed twice with 100 mM Tris–HCl buffer (pH 8.0). After washing, the cells were resuspended in 100 mM $NaHCO_3$–Na_2CO_3 buffer (pH 9.0) 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 purified with a His Gravitrap affinity column (GE Healthcare, Buckinghamshire, UK). The fractions containing protein were desalted with a PD-10 column (GE Healthcare, Buckinghamshire, UK) and eluted with 100 mM $NaHCO_3$–Na_2CO_3 buffer (pH 9.0).

Dipeptide synthesis using purified enzyme The standard reaction mixtures (300 μL) contained 20 mM Pro and 20 mM Gly, 40 mM Pro, or 40 mM Gly as substrates, 20 mM ATP, 20 mM $MgSO_4 \cdot 7H_2O$, and 0.5 mg/mL of the wild-type TabS or the mutants in 50 mM $NaHCO_3$–Na_2CO_3 buffer (pH 9.0). The reaction was performed at 30°C for 20 h, and stopped by heating 90°C for 10 min. The wild-type TabS or the mutants were removed by centrifugation (20,000 ×*g*, 30 min, 4°C). To evaluate the relationship between the amount of Pro–Gly synthesized and the concentration of the wild-type TabS or the mutants, we performed the reaction for 60 min with various concentrations of each enzyme (0.05–0.5 mg/mL) under standard conditions. To determine the kinetic parameters, we performed the reaction with various concentrations of Pro (20–400 mM), Gly (5–100 mM), or ATP (0.5–15 mM) under standard conditions (the enzyme concentration was 0.25 mg/mL), and the reaction time was 60 min.

Analysis The amounts of phosphate produced in reaction mixtures were measured with a Determiner L IP kit (Kyowa Medex, Tokyo) as the indicator of dipeptide synthesis. The amounts of dipeptides were analyzed by high performance liquid chromatography (HPLC) (L-2000 series; Hitachi High Technologies, Tokyo, Japan). The details of the analytical procedure were described previously (13). Data of HPLC analyses were averages from three independent experiments, and error bars indicated standard deviation of the means.

RESULTS

Evaluation of the mutants We chose to alter Ser85 and His294 residues of TabS that might position around the C- and N-terminal substrate and affect the enzyme activity. These residues were replaced with 20 proteogenic amino acids, and the dipeptide-synthesizing reactions were conducted using these mutants. The H294T and H294C mutants were not reacted because of their insolubilization. We measured the amount of phosphate released in the reaction mixtures to select the mutants that were able to synthesize Pro–Gly much more than the wild-type TabS (Fig. 2).

When Pro and Gly were used as substrate, the reaction mixtures of the S85T, S85C, S85M, S85V, S85L, and S85K mutants were contained phosphate equal to or more than that of the wild-type TabS (Fig. 2A). Under the same conditions, the reaction mixtures of the H294A, H294S, H294Q, and H294D mutants contained phosphate equal to or more than that of the wild-type TabS (Fig. 2B). These results indicated that 10 different mutants described above might have synthesized Pro–Gly considerably more than the wild-type TabS. Therefore, we measured the amount of Pro–Gly in the reaction mixtures of the mutants by HPLC (Fig. 3). The amount of Pro–Gly in the reaction mixture of the S85T and the H294D mutants was 1.2 and 1.4 times as much as that of the wild-type TabS, respectively. Therefore, we constructed the S85T/H294D double mutant.

Pro–Gly synthesis by the S85T/H294D double mutant We constructed the S85T/H294D double mutant to increase the amount of Pro–Gly expectantly. The dipeptide-synthesizing reaction was performed using 20 mM Pro and 20 mM Gly as substrates (Fig. 4). Gly–Pro, Gly–Gly, and Pro–Pro were synthesized in limited amounts by wild-type TabS and the mutants. The S85T/H294D double mutant synthesized Pro–Gly 1.6 times as much as the wild-type TabS, and considerably more than the S85T and the H294D mutant.

Characterization of the S85T/H294D double mutant First, we evaluated the relationship between the enzyme concentration and the amount of Pro–Gly (Fig. 5). This reaction was conducted for 60 min. Each enzyme concentration exhibited strong activity of the S85T/H294D double mutant. Next, we determined the kinetic