Screening of Salt Taste Enhancing Dipeptides and Effective Production of the Dipeptides by L-Amino Acid Ligase

L-アミノ酸リガーゼを利用した塩味増強効果を有するジペプチドの探索と効率的な合成法の開発

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Preface

Dipeptides have unique physiological functions including antihypertensive effects, sedative effects, and taste-improving effects. In this thesis, the author focused on taste-improving effects. Among them, the author especially searched for new salt taste enhancing dipeptides with the social back ground of increasing salt reduction-awareness.

In order to screen salt taste enhancing dipeptides, dipeptide library was constructed using L-amino acid ligase (Lal). Lal is a microbial enzyme that synthesizes dipeptides from unprotected L-amino acids. L-Methionylglycine and L-prolylglycine were found out as salt taste enhancers in the dipeptide library. Furthermore, effective production of the dipeptides was achieved using site-directed mutagenesis of Lals.

Industrial and academic knowledge of Lals was obtained in this thesis. The author believes that the results obtained from this thesis will open up the possibilities of dipeptides and Lals, and contribute to make dipeptides easier to synthesize than ever before.

Haruka Kino

Contents

Chapter 1

Review

Screening of Functional Dipeptides and Synthesis of Dipeptide by L-Amino Acid Ligase

1.1. Introduction

Dipeptides are composed of two amino acids joined by a peptide bond. Some dipeptides have unique physiological properties and physiological functions that their constitute amino acids do not show. Tasty or taste-improving effects are one of the functions of dipeptides. The author focused on the functions, especially the effect of salt taste enhancement. In recent years, concern has arisen about reducing salt intake. Salt (NaCl) is essential to the health of people; however, excessive salt intake increases blood pressure (1, 2) and causes diovascular disease (3). According to Health Japan 21 (second term), the target value of salt intake is 8.0 g/day. In contrast, National Health and Nutrition Survey of Japan in 2013 reported that the mean value of salt intake in adults was 10.2 g/day (men: 11.1 g/day and women: 9.4 g/day). Therefore, salt reduction is necessary, and the development of salt taste enhancing agents is expected. L-Leucyl-L-serine (Koike M, Japanese patent JP2012-165740, 2012), dipeptides containing arginine (4), and L-glutaminyl-L-threonine (5) have been reported as salt taste enhancing dipeptides. The author speculated other dipeptides have that effect and tried to find out salt taste enhancing dipeptides.

In this thesis, L-amino acid ligases (Lal, EC 6.3.2.28) were used to screen for salt taste enhancing dipeptides and to synthesize these dipeptides efficiently. Lal is an

enzyme that synthesizes dipeptides from unprotected L-amino acids and a member of the ATP-dependent carboxylate-amine/thiol ligase superfamily accompanying the hydrolysis of ATP to ADP (6, 7).

In this chapter, the author introduces the functions of dipeptides, screening methods of useful dipeptides, taste assessment methods, and characteristics of Lals.

1.2. Functional dipeptides

1.2.1. Tasty or taste-improving effect

When we take tasty dipeptides, they have sweet, umami, bitter, sour, salty, or other taste characteristics. Aspartame, which is a methyl ester of L-aspartyl-L-phenylalanine, is a representative tasty dipeptide and well known for chemical sweetener. It is widely contained in beverage, desserts, and tabletop sweetener and 200 times sweeter than sugar (sucrose) (8). L-Alanyl-L-histidine, glycyl-L-proline, glycyl-L-tryptophan, glycyl-L-valine, and L-leucyl-L-tryptophan have also sweet component, but they have possibly bitter taste (9). The most reported taste is bitter one. For instance, L-alanyl-L-leucine, L-alanyl-L-methionine, L-alanyl-L-phenylalanine, L-alanyl-L-serine, L-alanyl-L-tryptophan, L-alanyl-L-tyrosine, L-alanyl-L-valine, glycyl-L-aspartic acid, glycyl-L-glutamic acid, glycylglycine, glycyl-L-histidine, glycyl-L-leucine, glycyl-L-phenylalanine, glycyl-L-tyrosine, glycyl-L-isoleucine, leucyl-L-alanine, L-leucylglycine, L-leucyl-L-phenylalanine, L-leucyl-L-tyrosine, L-phenylalanyl-L-proline, L-phenylalanyl-L-valine, L-valylglycine, and L-valyl-L-valine are reported (9, 10). Some dipeptides exhibit sour taste as follows: glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-alanyl-L-aspartic acid, L-alanyl-L-glutamic acid, L-seryl-L-aspartic acid, L-seryl-L-glutamic acid, L-valyl-L-aspartic acid, L-valyl-L-glutamic acid, L-aspartyl-L-alanine, L-aspartic-L-aspartic acid, and so on (9, 10). There are reports of salt taste dipeptides,

L-alanyl-L-lysine HCl, glycyl-L-alanine, and L-leucyl-L-leucine, however, these dipeptides also have bitter taste or sour taste (9).

On the other hand, taste-improving dipeptides are not tasty themselves, but have the ability of enhancing or masking the taste. L-Glutaminyl- L-glutamic acid is known as a bitter-masking activity against various bitter substances such as L-isoleucine, glycyl-L-leucine, and caffeine (11). γ-L-Glutaminyl-L-glutamic acid, γ-L-glutaminylglycine, γ-L-glutaminyl-L-histidine, γ-L-glutaminyl-L-glutamine, γ-L-glutaminyl-L-methionine, and γ-L-glutaminyl-L-leucine from Gouda cheese show the ability of kokumi enhancing if NaOH or NaCl present (12). Kao Corp. demonstrates that L-Leucyl-L-serine is a salt taste enhancing dipeptide for low-salt soy sauce (Koike M, Japanese patent JP2012-165740, 2012). Schindler et al. find out salt taste enhancing dipeptides, containing arginine, in hydrolysate of fish protein (4). They evaluated the salt intensity of arginyl dipeptide in 50 mM NaCl solution and model broth solution adjusted 50 mM NaCl containing monosodium L-glutaminate monohydrate, maltodextrin, NaCl, and yeast extract, respectively. Some dipeptides exhibited almost the same salt intensity in any solution, but other dipeptides showed different salt intensity dependent on the solutions. For instance, L-arginyl-L-arginine did not exhibit salt taste enhancement effect in NaCl solution, but showed strong that effect in the model broth solution (4). Furthermore, L-glutaminyl-L-threonine has also salt taste enhancing effect and it shows that effect if bonito extract or L-arginine present (5).

1.2.2. Antihypertensive effect

The best known function of dipeptide is antihypertensive effects. Kagebayashi et al. demonstrated that L-arginyl-L-phenylalanine exhibits vasorelaxaing and antihypertensive activity (13). They suggested that sequence of L-arginyl-L-phenylalanine is important. In fact,

L-isoleucyl-L-histidyl-L-arginyl-L-phenylalanine from rice albumin exhibits vasorelaxaing and antihypertensive activity, though L-arginine, L-phenylalanine, and L-phenylalanyl-L-arginine have no such function (13). L-Isoleucyl-L-tryptophan shows also anti antihypertensive activity (14). L-Isoleucyl-L-tryptophan was gained from salmon peptide, digestion of salmon muscle, and it has not vasorelaxaing activity but strong inhibitory activity against the angiotensin I-converting enzyme (ACE) (14). L-Tyrosyl-L-proline also has inhibitory activity of ACE and was produced by fermenting using *Lactobacillus helveticus* in skim milk medium (15). Furthermore, there are many patents related to dipeptides of ACE inhibitor. For example, Kikkoman Corp. developed soy source containing glycyl-L-tyrosine and L-seryl-L-tyrosine that exhibits ACE inhibitory activity (Endo Y, WO 2011/078324 A1, 2011). Yamaki Corp. found that L-seryl-L-tryptophan, L-aspartyl-L-tryptophan, L-glutamyl-L-tryptophan, glycyl-L-tryptophan, and L-alanyl-L-tryptophan in fish hydrolysate have the effect (Seki E, Asada H, Japanese patent 5456144, 2014).

1.2.3. Other effects

L-Tyrosyl-L-leucine has anxiolytic-like activity and its activity is stronger than diazepam that was medicine for the relief of symptom related to anxiety disorders (16). Interestingly, L-tyrosine and L-leucine have no such function (16). Takagi et al. discovered L-tyrosyl-L-arginine (Kyotorphin), which have analgesic effect, from bovine brain (17). L-Seryl-L-histidine and L-isoleucyl-L-histidine also show sedative effects (18). There is a report about antioxidant peptides from hydrolysate of dried bonito, and L-lysyl-L-aspartic acid is contained in it (19). Furthermore, L-isoleucyl-L-tryptophan exhibits the inhibitory activity of human dipeptidyl peptidase IV that is used as treating agent for type 2 diabetes (20).

1.3. Screening methods for functional dipeptides

Many functional dipeptide described above are found from proteolytic or microbial digests of natural proteins. For instance, to identify salt taste enhancing dipeptides, Schindler et al. digested fish protein by chymotrypsin, and the digests were separated by ultrafiltration. After the low molecular weight fraction was separated by gel permeation chromatography, sensory test was conducted for each fraction and determined the fraction containing salt taste modulating (STM) peptides. Finally, STM peptides were identified in candidate fraction (4). Suetsuna digested molsin to detect antioxidant dipeptides. The digests were separated by ion-exchange chromatography and gel filtration. The fraction that showed antioxidant activity was further separated by HPLC, and finally 11 peptides were identified (19). ACE-inhibitory dipeptide L-tyrosyl-L-proline was detected in microbial digestion of skim milk. Digestion by *Lactobacillus helveticus* CPN4 was fractionated, and ACE-inhibitory activities of each fraction were measured. The fraction with the highest activity was separated repeatedly, and L-tyrosyl-L-proline was determined as the ACE-inhibitory dipeptide because of containing only one dipeptide in the final fraction (15). On the other hand, a few functional dipeptides were found out from not digests but dipeptide library. For instance, L-isoleucyl-L-tryptophan, the inhibitor of human dipeptidyl peptidase IV, was found from the library. This assay uses 96-well plates, and high-throughput screening is possible to be conducted (20).

1.4. Evaluation methods for tasty or taste-improving agents

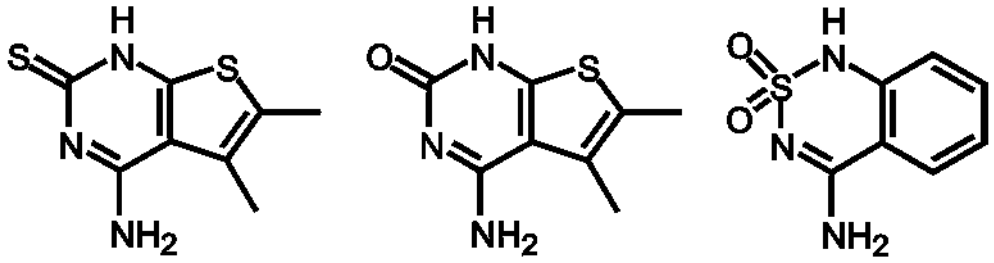
1.4.1. Sensory assessment

Sensory assessment is best-known assessment of tastes, and food industry has long relied on the assessment. Professional panelists evaluate food, chemicals, drugs, and so on within well-controlled procedures (21). There are many well-established

methods such as discrimination tests, scaling tests, expert tasters, affective tests, and descriptive methods (21). Sensory tests are the main methods of evaluating tastes in the food industry; however, there are problems of need for training panelists, toxicity, low through put, and ethical issues (21). In addition, senses for taste substances are affected by age, and dietary habits, palatability, physical condition, and emotional state (22).

1.4.2. Objective taste assessment: Utilization of taste receptor

Five basic tastes, sweet, umami, bitter, sour, and salty are mediated by each taste receptors in taste buds on tongue (23). Taste receptors generate action potentials and release neurotransmitter according to information of taste substances (24). We are able to recognize taste through this mechanism. Studies of four basic tastes except for salty have progressed in particular. Sweet, umami, and bitter taste are mediated by a family of G-protein-coupled receptors, and bitter taste is mediated by an ion channel receptor (23). Taste assessments on the basis of taste receptors were constructed and screening of the taste-improvement agents was conducted (25). For instance, to identify molecules which affect the tastes, receptor-based assays have been reported. Servant et al. showed that SE-1, SE-2, and SE-3 (Fig. 1.1) were sweet enhancers through screening using a cell-based assay for the human sweet taste receptor (T1R2 and T1R3) (26). They also reported that these molecules had not sweet taste themselves.



(A) (B) (C)

Fig. 1.1. The structures of SE-1 (A), SE-2 (B), and SE-3 (C)

Sakurai et al. used human bitter taste receptor (hTAS2R16) in order to evaluate the bitter-masking dipeptides such as L-glutaminyl-L-glutamic acid (Glu-Glu) and L-aspartyl-L-aspartic acid (27). Glu-Glu was contained in acidic fraction of fish hydrolysate and it showed bitter-masking activity for bitter substances (28). Kim et al. demonstrated the interaction between bitter and umami taste using hTAS2R16 (29). In contrast, we do not yet know everything about salt taste receptor mechanism, and salt taste enhancers have not been found out using salt taste receptor. The study of the mechanism has been gradually advanced. Oka et al. elucidated that low concentration of NaCl activate the epithelial sodium channel (ENaC) (30) and high concentration of NaCl activate the bitter taste and sour taste receptors in addition to ENaC (31). Furthermore, Lu et al. reported that S3969 (Fig. 1.2) is the activator of the human ENaC (32). Cell-based assay that was used to screen sweet enhancers such as SE-1, SE-2, and SE-3 (Fig. 1.1) has been developed recently, and it can evaluate more samples at short time than ever before (25). For these achievements and characteristics, utilization of taste receptor is useful assessment if the laboratory equipment is completed.

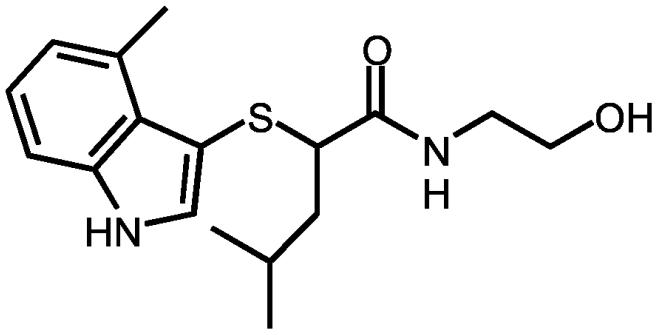


Fig. 1.2. The structure of S3969.

1.4.3. Objective taste assessment: Utilization of taste sensor

Taste sensor is composed of several kinds of lipid/polymer membranes and

information of taste substances converts into electric signal (33). Taste sensor analysis does not measure the amount of specific taste substances but is able to grasp taste characteristics as the sensory assessment (33). Bleibaum et al. demonstrated correlation between evaluation by consumers and taste sensor for apple juices (34). Ito et al. showed correlation between predicted bitter taste scores using taste sensor and actual bitter taste scores using sensory assessment (35). They evaluate masking effect of artificial sweeteners on the bitter taste of H_1-antihistamines using taste sensor (35). Besides this, there are reports about evaluations of various foodstuffs using taste sensor (36-39). In sensory assessment, senses for taste substances are affected by age, physical condition, and emotional state. Contrary to this, taste sensor analysis is not affected by such elements (22). In addition, taste sensor analysis is conducted automatically, and special techniques do not need to operate taste sensor system. For these achievements and characteristics, taste sensor analysis has been used as objective and convenient taste assessment.

1.5. L-Amino acid ligase

1.5.1. Characteristics of Lals

As mentioned previously, Lal is an enzyme that synthesizes dipeptides from unprotected L-amino acids, and the reaction accompanies the hydrolysis ATP to ADP (Fig. 1.3) (7). About 20 kinds of Lals have been reported, and each Lal has unique substrate specificity. Tabata et al. conducted in silico screening and found YwfE from

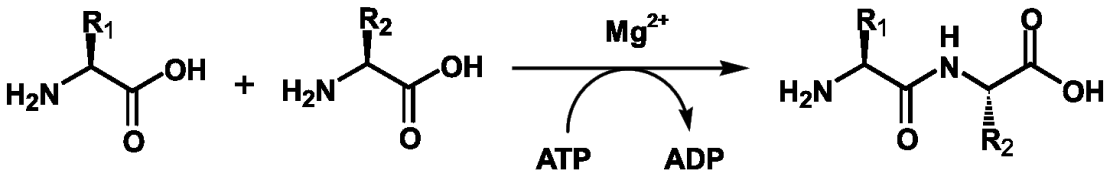


Fig. 1.3. Dipeptide synthesis catalyzed by Lal.

Bacillus subtilis 168 in 2005 (7). It was the first report of Lal. YwfE prefers nonbulky small amino acids such as glycine, L-alanine, and L-serine as the N-terminal substrate and prefers bulky and neutral amino acids such as L-phenylalanine, L-methionine and

		C-terminal amino acid														
		Gly	Ala	Ser	Cys	Thr	Val	Leu	Ile	Met	Phe	Tyr	Trp	Gln	Asn	His
N-terminal amino acid	Gly	●			●			●		●	●	●		●		
	Ala	●	●	●	○	●	●	●	●	●	●	●	●	●	●	●
	Ser	●	●	●	○		●	●	●	●	●	●	●	●		●
	Thr	●				○		●		●	●			●		
	Met									●	●	●				●
	Cys			○	○	○		○	○	○	○		○			○

Fig. 1.4. Substrate specificity of YwfE (7).
Reaction mixtures were analyzed by HPLC. A filled circle showed that corresponding dipeptide was synthesized by HPLC. An open circle showed that new peak was confirmed by HPLC.
Reaction mixtures contained 15 mM Xaa1, 15 mM Xaa2, 60 mM ATP, 30 mM $MgSO_4$, and 0.05 mg/mL TabS in 50 mM Tris-HCl buffer (pH 9.0) . The reaction was performed at 37ºC for 12 h. Amino acids are written in three letter codes.

L-leucine as the C-terminal substrate (Fig. 1.4, Xaa showed any amino acid) (7). Furthermore, L-alanyl-L-glutamine was synthesized through fermentative process using metabolically engineered *Escherichia coli* expressing YwfE (40). Discovery of YwfE led to identification of other Lals such as RSp1486a from *Rastonia solanacearum* JCM19498 (41), BL00235 from *Bacillus licheniformis* NBRC1220042), RizA and RizB from *Bacillus subtilis* NBRC3134 (43, 44), and TabS from *Pseudomonas syringae* NBRC14081 (45). RSp1486a accepts L-asparagine and L-glutamine for only N-terminal substrate and accepts glycine, L-leucine, L-threonine, and L-valine for only C-terminal substrate (41).The amino acids as C-terminal substrate in Rsp1486a have little in common with structure. BL00235 has also strict substrate specificity, and only L-methionine and L-leucine are acceptable for the N-terminal amino acid (42). RizA

synthesizes dipeptides that contain only L-arginine as the N-terminal substrate (43). On the other hand, TabS has broad substrate specificity, and dipeptides are detected in 136 of 231 reaction mixtures containing one or two amino acids which are selected from 20 proteogenic amino acids and β-alanine as substrates (Fig. 1.5) (45). Furthermore, TabS has distinctive substrate selectivity toward N- and C-terminal substrate unlike other

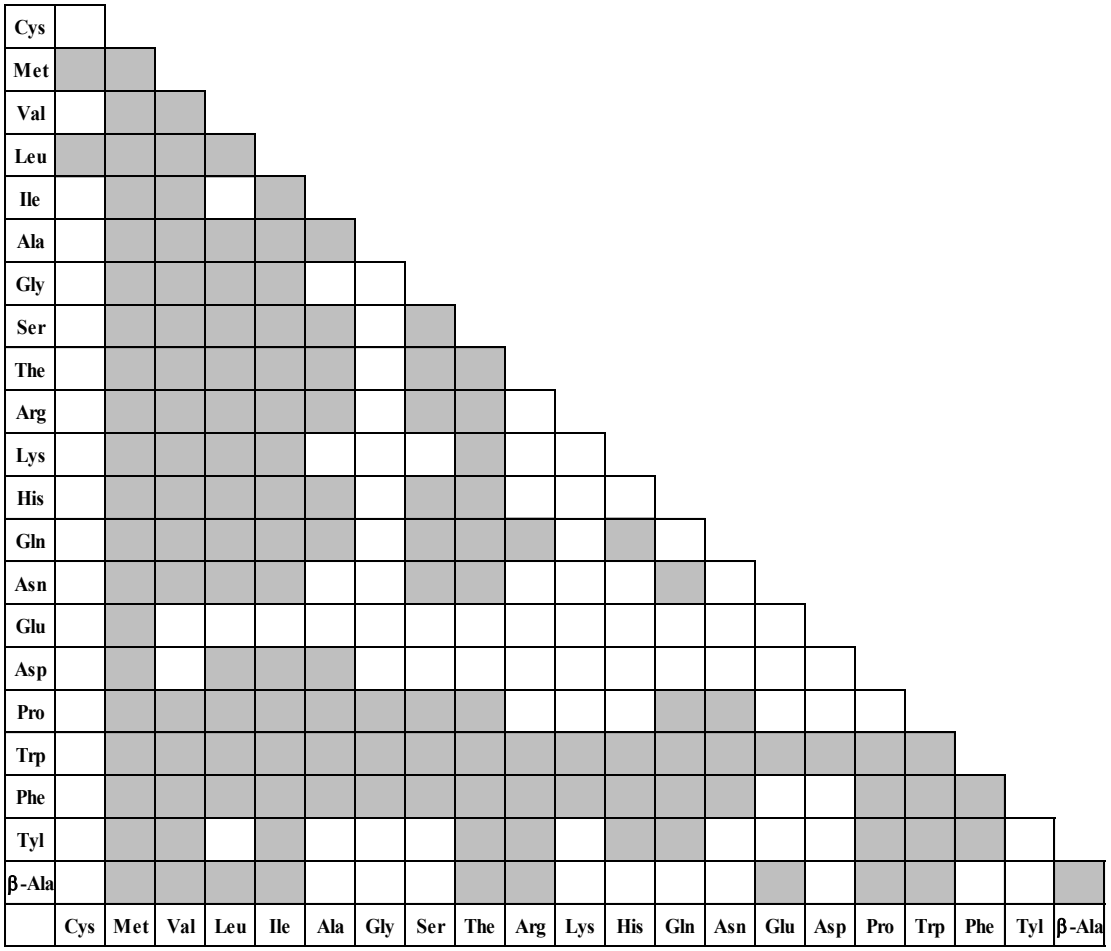


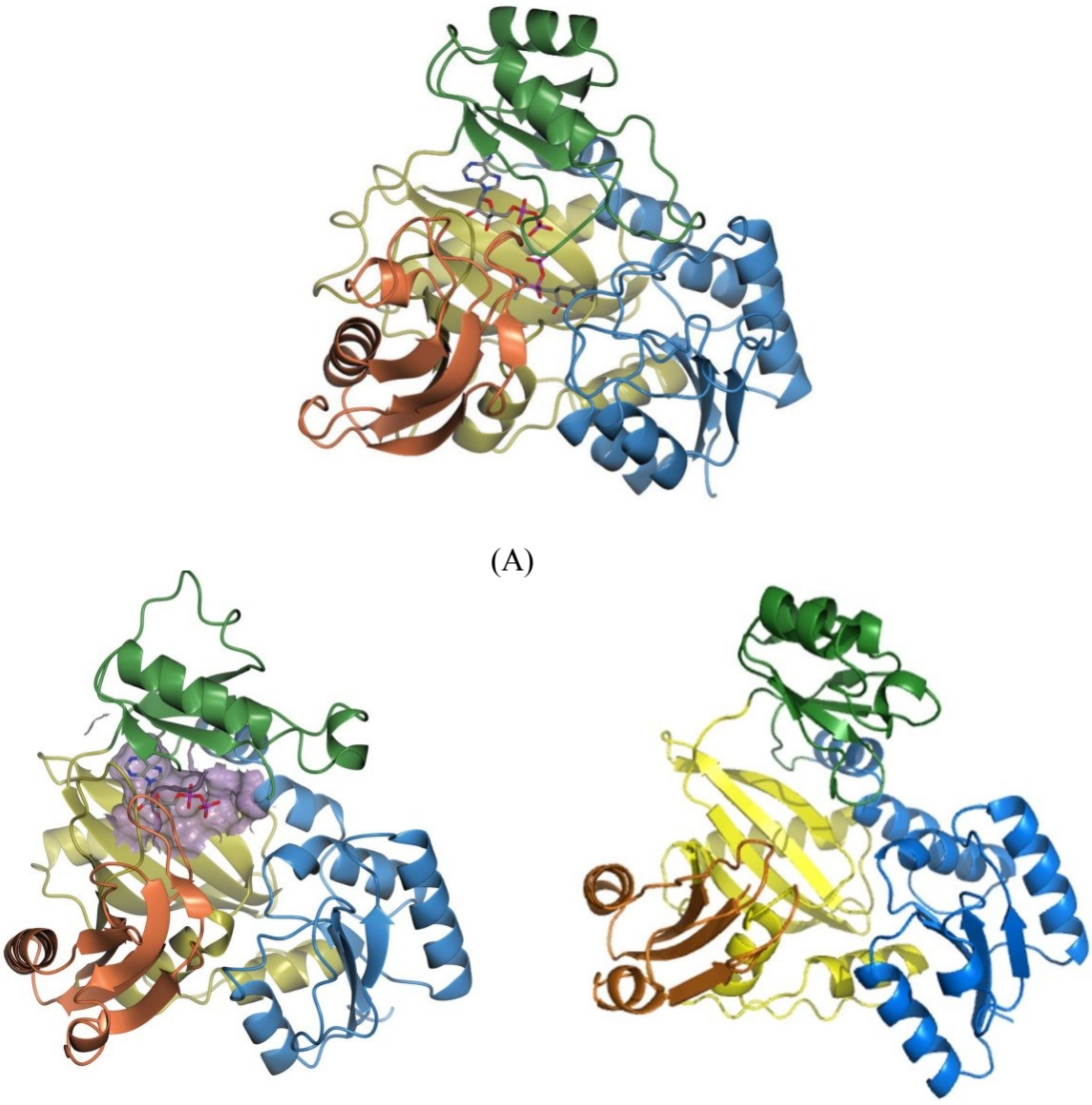
Fig. 1.5. Overview of the substrate specificity of TabS (45**).**
Reaction mixtures were analyzed by LC-ESI MS. A filled column indicated the formation of the corresponding dipeptide.
Reaction mixtures contained 12.5 mM Xaa1, 12.5 mM Xaa2, 12.5 mM ATP, 12.5 mM $MgSO_4$, and 0.1 mg/mL TabS in 100 mM Tris-HCl buffer (pH 9.0). The reaction was performed at 30ºC for 20 h. Amino acids are written in three letter codes.

Lals, and TabS is able to synthesize useful dipeptide such as L-leucyl-L-isoleucine (antidepressive effect), L-arginyl-L-phenylalanine (antihypertensive effect) and L-leucyl-L-serine (enhances saltiness) in high yield (45). These Lals described above synthesize only dipeptides. Some Lals catalyze oligopeptides synthesis. RizB is the first reported Lal that synthesizes oligopeptides and has the highest activity toward L-valine, L-leucine, L-isoleucine, and L-methionine (44). Arai et al. conducted in silico screening using the amino acid sequence of RizB as query and obtained BL02410, Haur_2023, spr0906, BAD_1200 and CV_0806 that synthesize oligopeptides (46). These Lals prefer L-valine, L-leucine, L-isoleucine, and L-methionine (Fig. 1.6) (46).

Enzyme	Substrate	Product					Enzyme	Substrate	Product				
		2mer	3mer	4mer	5mer	6mer			2mer	3mer	4mer	5mer	6mer
RizB	Val	●	●	●	●		BL002410	Val	●	●	●	●	
	Leu	●	●	●				Leu	●	●	●	●	
	Ile	●	●	●				Ile	●	●	●		
	Met	●	●	●	●			Met	●	●	●	●	
	Trp	●						Trp	●	●			
	Phe							Phe	●	●			
	Tyr							Tyr					
Haur_2023	Val	●	●	●			spr0906	Val	●	●	●	●	●
	Leu	●	●	●				Leu	●	●	●	●	●
	Ile	●	●					Ile	●	●	●	●	
	Met	●	●	●				Met	●	●	●	●	●
	Trp	●						Trp	●				
	Phe	●	●					Phe	●				
	Tyr							Tyr					
BAD_1200	Val	●	●	●	●		CV_0806	Val	●	●	●		
	Leu	●	●	●	●	●		Leu	●	●	●		
	Ile	●	●	●				Ile	●				
	Met	●	●	●	●	●		Met	●	●			
	Trp	●	●	●	●			Trp	●				
	Phe	●	●	●	●			Phe					
	Tyr	●	●	●	●			Tyr					

Fig. 1.6. Oligopeptide synthesis using Lals (46).
Reaction mixtures were analyzed by LC-ESI MS. A filled circle indicated the formation of the corresponding dipeptide.
Reaction mixtures contained 25 mM Xaa, 12.5 mM ATP, 12.5 mM $MgSO_4$, and 0.1 mg/mL Lal in 100 mM Tris-HCl buffer (pH 8.0). The reaction was performed at 30ºC for 20 h. Amino acids are written in three letter codes.

1.5.2. Structures of Lals



(A)

(B) (C)

Fig. 1.7. The overall structures of YwfE (A), BL00235 (B), and RizA (C)
The A-domain, B-domain, C1-domain, and C2-domain are shown in blue, green, gold, and orange, respectively. The figures were prepared using PyMol.

Only three structures of Lals have been reported so far (Fig. 1.7); YwfE (PDB ID: 3VMM), BL00235 (PDB ID: 3VOT) and RizA (PDB ID: 4WD3) (47-49). The overall structures are highly similar, though the amino acid sequence of each Lal shows low homology. The homology between YwfE and BL00235 is 23.6%, YwfE

and RizA is 21%, and RizA and BL00235 is 20% (42, 43). The structures of Lals are composed of four domains, the A-domain, B-domain, C1-domain, and C2-domain. YwfE is solved in complex with ADP and an intermediate analog, phosphorylated phosphinate L-alanyl-L-phenylalanine (P-analog) (47). BL00235 is solved in complex with ADP, and RizA is crystallized with the substrate-free form (48, 49). The substrate recognition site of YwfE and BL00235 are shown Fig. 1.8. When the amino acid residues of which position around N-terminal substrate of BL00235 are focused, Tyr238, Val296, Ile340, Tyr386 and Phe389 residues form a hydrophobic cavity (Fig. 1.7 (B)). For this cavity, BL00235 accepts only L-leucine and L-methionine for the N-terminal substrate (48). In contrast, this cavity of YwfE is filled with Trp332 and Met334 which have bulky hydrophobic side chains (Fig. 1.7 (A)), and YwfE prefers small amino acid such as glycine, L-alanine, L-serine, and L-threonine owing to the narrow space around the N-terminal substrate (48). BL00235 prefers glycine, L-alanine, and L-serine which have small side chain as the C-terminal substrate (42). The C-terminal substrate recognition site of BL00235 indicates that Phe83 and Pro85 residues occupy the space around C-terminal substrate, and these residues prevent the bulky amino acid residues from entering the pocket as C-terminal substrates (48).

1.5.3. Recent studies of Lals

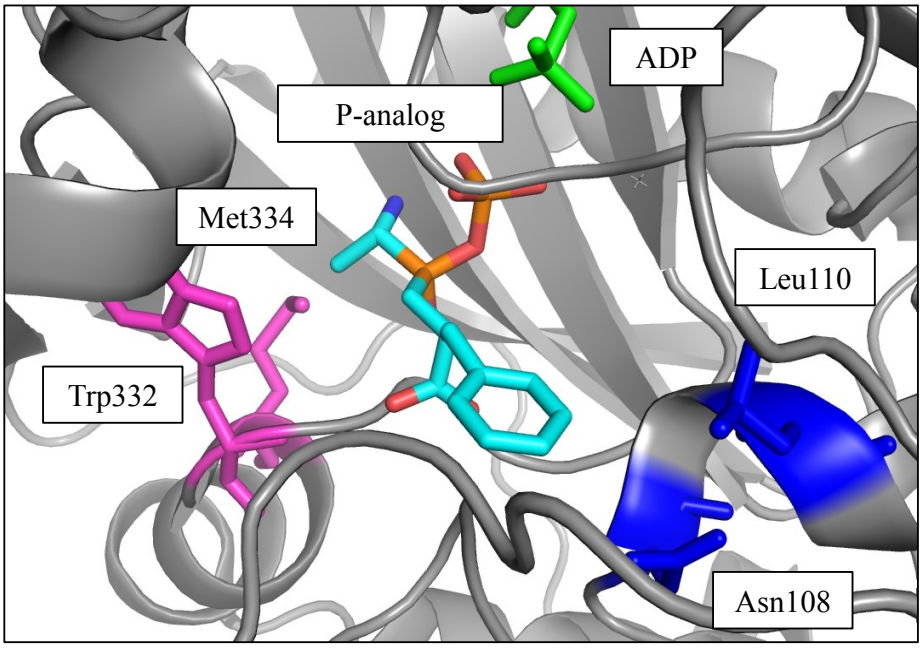
Lal was studied so far with a focus on findings new Lals (40-46), and determination crystal structures and alteration of the substrate specificities have been increased recently. Only a few reports about the alteration of Lals with structure-based site-directed mutagenesis have been published (47, 50). The objectives of mutant construction can be roughly divided into two types: (i) to reveal the catalytic function (47, 50) and (ii) to alter substrate specificity in order to synthesize useful dipeptides (50). Tsuda et al. indicated that Trp332 mutants of YwfE are able to alter the substrate

specificity and suggested that alteration of substrate specificity of Lal might have led to synthesize desirable dipeptides (50). However, no study has demonstrated that changing the substrate specificity of Lals by a single mutation on the basis of the structures made the mutants synthesize dipeptides as planned.

1.6. Objective of this thesis

This thesis is composed of two major parts. One is screening for salt taste enhancing dipeptides using Lal, and the other is effective production of the dipeptides. Dipeptides have many functions, and one of them is taste-improving effects described above (4, 5, 8-12). In addition, salt deduction is necessary for us with social background of health-conscious, and the development of salt replacers or salt taste enhancing agents is expected. Therefore, the author speculated that some dipeptides have salt taste enhancing effect among functional unknown dipeptides. New screening method was constructed using Lals, and new salt taste enhancing dipeptides were found. Furthermore, the author altered the substrate specificity of Lals to synthesize these dipeptides efficiently including selective synthesis. This thesis is the first report of succeeding in synthesizing useful dipeptide efficiently using Lals by site-directed mutagenesis.

(A)



(B)

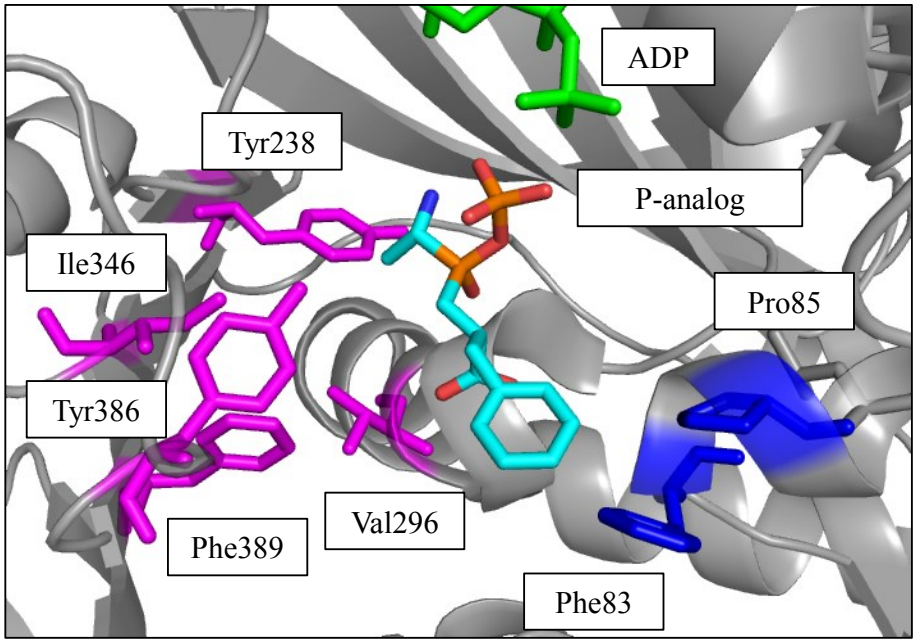


Fig. 1.8. The substrate recognition site of YwfE (A) and BL00235 (B).
ADP (green), the P-analog (C atoms in light blue), and some residues were drawn with stick models. The figures were prepared using PyMol.
BL00235 was shown as superimposed model complex with P-analog of YwfE.

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Chapter 2

Screening of Salt Taste Enhancing Dipeptide Using L-Amino Acid Ligase

2.1. Introduction

Dipeptides are composed of two amino acids joined by a peptide bond. Some dipeptides have unique physiological properties and physiological functions that their constitute amino acids do not show. For example, L-isoleucyl-L-tryptophan (1), L-arginyl-L-phenylalanine (2), and L-tyrosyl-L-proline (3) have antihypertensive effects, and L-seryl-L-histidine (4) and L-isoleucyl-L-histidine (4) have a sedative effect. Furthermore, some dipeptides have tasty or taste-improving effect. Aspartame, which is a methyl ester of L-aspartyl-L-phenylalanine, is well known for chemical sweetener. It is widely contained in beverage, desserts, and tabletop sweetener, and its sweetness is 200 times stronger than sugar (sucrose) (5). In addition, Schiffman SS et al. reported that L-alanyl-L-histidine, glycyl-L-proline, glycyl-L-tryptophan, glycyl-L-valine, and L-leucyl-L-tryptophan were bitter and sweet (6). Except for sweet, L-alanyl-L-aspartic acid and L-alanyl-L-glutamic acid are sour-bitter, and L-alanyl-L-leucine and L-alanyl-L-methionine are bitter (6). L-Alanyl-L-lysine HCl, glycyl-L-alanine, and L-leucyl-L-leucine are salty, however, these dipeptides also have bitter taste or sour taste (6). Furthermore, L-leucyl-L-serine (Leu-Ser) (Koike M, Japanese patent JP2012-165740, 2012), dipeptides containing arginine (Arg) (7), and L-glutaminyl-L-threonine (8) have the effect of salt taste enhancement.

In recent years, concern has arisen about reducing salt intake. Salt (NaCl) is essential to the health of people; however, excessive salt intake increases blood pressure (9, 10) and causes diovascular disease (11). According to Health Japan 21 (second term), the target value of salt intake is 8.0 g/day in 2015. In contrast, National Health and Nutrition Survey of Japan in 2013 reported that the mean value of salt intake in adults was 10.2 g/day (men: 11.1 g/day and women: 9.4 g/day). Therefore, salt reduction is necessary, and the development of salt replacers or salt taste enhancing agents is expected. Salt Institute (Virginia, USA) listed these agents (12) (Table 2.1.). Potassium chloride which is known as the salt replacer has severe off-taste (7). There is a doubt about the effect of L-ornithyl-β-alanine (7). The other agents do not have strong effect of salt taste enhancement.

Table 2.1. Salt taste enhancing agents and salt replacers (12).

Salt taste enhancers	Salt replacers
5-ribonucleotides	Potassium chloride
Glycine monoethyl estel	Calcium chloride
L-Lysine	Magnesium sulfate
L-Arginine	Various metal ion replacers
Lactates	
Mycosent	
Monosodium glutamate	
Trehalose	
L-Ornithyl-β-alanine	
L-Ornithine	
Alapyridaine (N-(1-Carbixethyl)-6-hydroxymethyl-pyridinium-3-ol)	

There are some dipeptides such as Leu-Ser which have effect of salt taste enhancement as described above. The author deduced that other dipeptides have also this effect. Most of functional dipeptides are derived from proteolytic (1, 4, 7, 8, 13) or microbial (3) digests of natural proteins. The author speculated that many dipeptides digested easily by the hydrolysis of natural proteins could not be remaining in the dipeptides and have not been evaluated of their functions so far. Therefore, these dipeptides were synthesized by L-amino acid ligase (Lal) and evaluated by sensory assessment.

Lal is an enzyme that synthesizes dipeptides from unprotected L-amino acids, and is a member of the ATP-dependent carboxylate-amine/thiol ligase superfamily accompanying the hydrolysis ATP to ADP (14, 15). About 20 kinds of Lals have been found out, and each Lal has unique substrate specificity (16-19). In this chapter, the author synthesized dipeptides using TabS from *Pseudomonas syringae* NBRC14081, which has the broadest substrate specificity of any known Lal (19). Furthermore, two-step screening system was constructed, and the candidates of new salt taste enhancing dipeptides were found out.

The contents in this chapter were summarized in the research paper (20). Amino acids are written in three letter code as follows; glycine (Gly), L-alanine (Ala), L-serine (Ser), L-threonine (Thr), L-cysteine (Cys), L-methionine (Met), L-valine (Val), L-leucine (Leu), L-isoleucine (Ile), L-arginine (Arg), L-lysine (Lys), L-histidine (His), L-glutamine (Gln), L-asparagine (Asp), L-glutamic acid (Glu), L-asparatic acid (Asp), L-tryptophan (Trp), L-phenylalanine (Phe), L-tyrosine (Tyr), L-proline (Pro).

2.2. Materials and Methods

2.2.1. Materials

All chemicals used in this study are commercially available and are of

chemically pure grade.

2.2.2. Enzyme preparation

The genes encoding TabS were previously cloned into the pET28a(+) vectors (19). Recombinant *Escherichia coli* BL21 (DE3) cells were cultivated in 3 mL LB medium (1% bacto tryptone, 0.5% yeast extract, 1% NaCl) supplemented 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 on a gyratory shaker (120 rpm) at 37°C. After cultivating for 1 h, 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to the medium, and cultivation conducted at 25°C for an additional 19 h with shaking on a gyratory shaker (120 rpm). The cells were collected with centrifugation (3,000 × g, 10 min, 4°C) and washed twice with 100 mM Tris-HCl buffer (pH 8.0). After washing, the cells were suspended in 100 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0) and then lysed by sonication at 4°C. The lysate was centrifuged (20,000 × g, 30 min, 4°C), and the supernatant was purified and fractionated with a His Gravitrap^{TM} 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).

2.2.3. Dipeptide synthesis using purified enzyme

The standard reaction mixtures (21 mL) contained 20 mM amino acid substrates, 20 mM ATP, 20 mM $MgSO_4 \cdot 7H_2O$, and 0.5 mg/mL of TabS 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. TabS was removed by centrifugation (20,000 × g, 20 min, 4°C).

2.2.4. Analysis

The amounts of phosphate produced in reaction mixtures were measured with a Determiner L IP kit (Kyowa Medex, Tokyo, Japan) as the indicator of dipeptide synthesis. The amounts of dipeptides were analyzed by HPLC (L-2000 series; Hitachi High Technologies, Tokyo, Japan). The details of the analytical procedure were described previously (21).

2.2.5. Sensory assessment

In the first screening, 0.60% (w/v) NaCl solution containing 40% (w/v) reaction mixture (final concentration) as the test sample and 0.60% (w/v) NaCl solution containing 0.10% (w/v) Leu-Ser and 0.40% (w/v) ATP (final concentration) as the control sample were prepared. Five panelists tested 0.5 mL of each sample. The test samples of which salt taste intensities were equal to or stronger than that of the control sample evaluated by three more panelists were submitted for the second screening. Furthermore, the evaluations were scored on one point (salt intensity of the test sample was equal to that of the control sample) or two point (salt intensities of the test sample was stronger than that of the control sample). Before the second screening, the amounts of residual amino acids in the reaction mixtures were measured by HPLC.

In the second screening, 0.60% (w/v) NaCl solution containing 40% (w/v) reaction mixture (final concentration) as the test sample and 0.60% (w/v) NaCl solution containing 40% (w/v) amino acids solutions and 0.40% (w/v) ATP (final concentration) as the control sample were prepared. Amino acids solutions contained the same amount of amino acids as the corresponding reaction mixture did. Five panelists tested 0.5 mL of each sample. The test samples of which salt taste intensities were stronger than that of the control sample evaluated by three more panelists were candidates for the reaction mixtures containing salt taste enhancing dipeptides.

2.3. Results

2.3.1. Dipeptide library construction using TabS

Seven amino acids, Leu, Phe, Ser, Val, Arg, Met and Gly, which were easily released by the hydrolysis of proteins or peptides (22-24), were selected, and 111 kinds of reaction mixtures containing mainly these amino acids as substrates were prepared. The amounts of phosphate produced in the reaction mixtures were measured (Fig. 2.1). When Leu, Ser, Val, and Met were used as substrates, phosphate released in the reaction mixture was more than 5 mM, TabS had high activity toward these amino acids. In contrast, when Phe, Arg, and Gly were used as substrates, released phosphate was less than 5 mM depending on the combination of substrates.

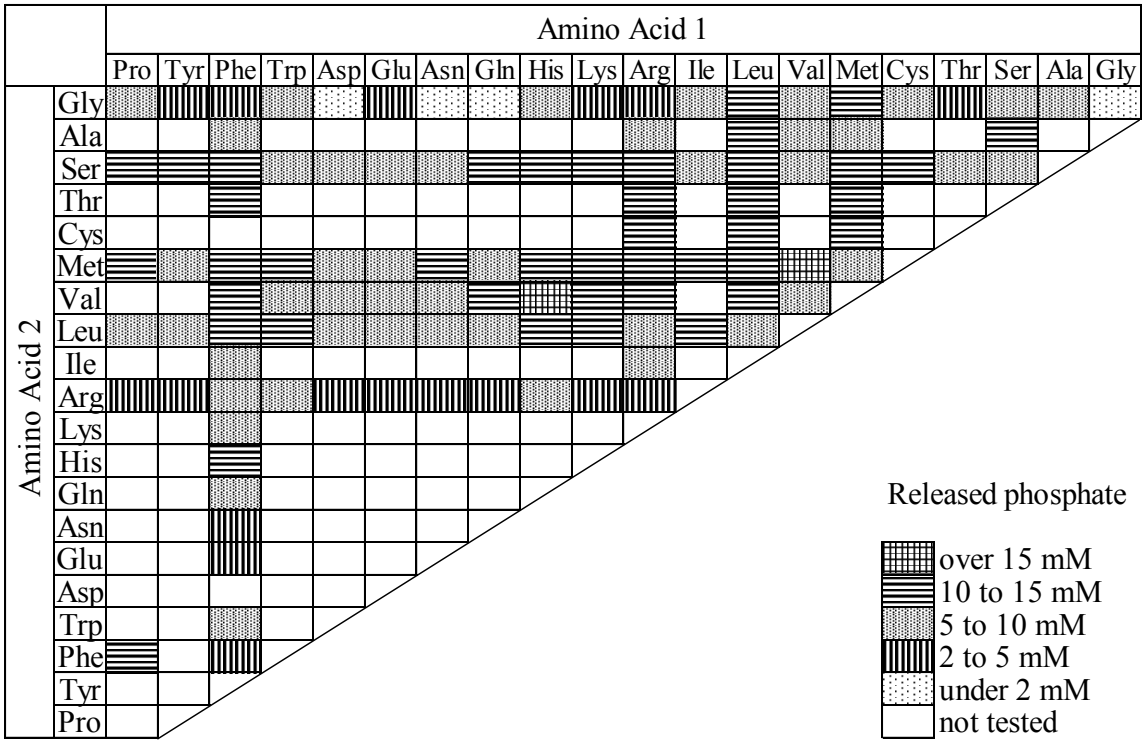

Fig. 2.1. Measurement of the amounts of phosphate produced in the reaction mixtures.
Reaction mixtures contained 20 mM Xaa1, 20 mM Xaa2, 20 mM ATP, 20 mM $MgSO_4$, and 0.5 mg/mL TabS in 50 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0). The reaction was performed at 30ºC for 20 h.

2.3.2. Sensory assessment

2.3.2.1. First screening

The reaction mixture contained not only dipeptide synthesized by TabS but also components such as ATP and residual amino acids which affect taste. To exclude the effect of these factors, two-step screening system was constructed. In the first screening, ATP and Leu-Ser was added to the control sample to exclude from influence of ATP contained in the test sample. Five panelists compared salt taste intensities between control sample and test samples, and 17 kinds of test samples of which salt taste intensities were equal to or stronger than that of the control sample were selected (Table 2.2). All panelists judged that three reaction mixtures, combinations of Leu and Ser, Arg only, and Arg and Tyr, were salty. In particular, the reaction mixture containing only Arg as substrate obtained full marks (10 points).

2.3.2.2. Second screening

In the second screening, amino acids and ATP were added to the control sample to exclude the influence of residual amino acids. The amounts of residual amino acids in the 17 kinds of candidate reaction mixtures were measured by HPLC (Fig. 2.3). Each amino acids solution added to the control samples was prepared based on these concentrations. Five panelists compared salt taste intensities between control sample and test sample, and eight kinds of test samples of which salt taste intensities were stronger than that of the control sample were selected (Table 2.3). The author considered that dipeptides contained in these reaction mixtures were salt taste enhancers. Among them, the author focused on two reaction mixtures, combinations of Met and Gly, and Pro and Gly, because the other six reaction mixtures contained predictive dipeptides such as Leu-Ser (Koike M, Japanese patent JP2012-165740, 2012) and Arg-Lys (7) that had already reported as salt taste enhancers. In order to determine the salt taste enhancing dipeptide contained in the two reaction mixtures,

qualitative and quantitative analysis of the reaction mixtures were conducted by HPLC analyses. When Met and Gly were used as substrates, 3.7±0.047 mM L-Met-Gly (Met-Gly) and 6.1±0.25 mM L-Met-L-Met (Met-Met) were synthesized. Although Met-Met was also synthesized in the reaction mixture contained Met only as a substrate, this reaction mixture did not exhibit the effect of salt taste enhancement with first screening. On the other hand, when Pro and Gly were used as substrates, TabS synthesized 8.3±0.32 mM Pro-Gly and hardly synthesized few other three dipeptides. Therefore, the author assumed that Met-Gly and Pro-Gly were new salt taste enhancing dipeptide.

Table 2.2. First screening.

Amino acid 1	Amino acid 2	Number of panelists (persons) [a]	Score (point) [a]
Leu	Ser	5	6
Leu	Glu	3	5
Met	Gly	3	3
Met	Arg	3	3
Met	Lys	3	3
Arg	Gly	3	4
Arg	Ala	3	4
Arg	Thr	3	4
Arg	Ile	3	4
Arg	Arg	5	10
Arg	His	3	3
Arg	Tyr	5	8
Arg	Gln	4	5
Arg	Lys	4	6
Arg	Asp	3	6
Arg	Asn	3	4
Pro	Gly	3	3

[a] Number of panelists judging that salt taste intensities of test sample was equal to (one point) or stronger than that of the control sample (two points).

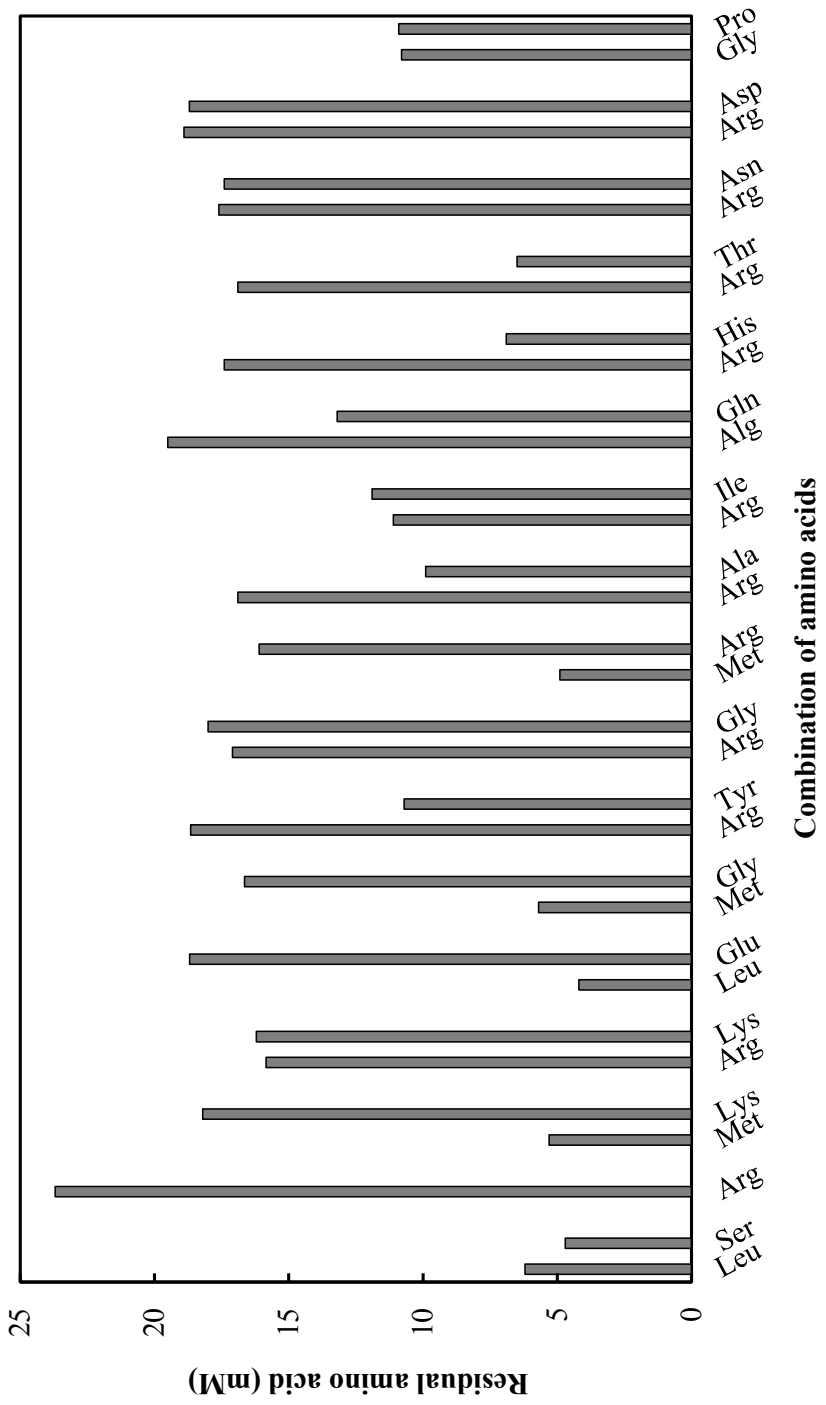

Fig. 2.2. Measurement of the amounts of residual amino acids in the reaction mixtures.

Reaction mixtures contained 20 mM Xaa1, 20 mM Xaa2, 20 mM ATP, 20 mM $MgSO_4$, and 0.5 mg/mL TabS in 50 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0). The reaction was performed at 30ºC for 20 h.

Table 2.3. Second screening.

Amino acid 1	Amino acid 2	Number of panelists (persons) [a]
Leu	Ser	4
Leu	Glu	1
Met	Gly	4
Met	Arg	2
Met	Lys	2
Arg	Gly	4
Arg	Ala	1
Arg	Thr	2
Arg	Ile	0
Arg	Arg	2
Arg	His	3
Arg	Tyr	1
Arg	Gln	1
Arg	Lys	3
Arg	Asp	3
Arg	Asn	3
Pro	Gly	3

[a] Number of panelists judging that salt taste intensities of test sample was stronger than that of the control sample.

2.4. Discussion

In this chapter, dipeptides were synthesized by Lal and two-step screening system was constructed using the reaction mixtures to screen for salt taste enhancing dipeptides. Many functional dipeptides were derived from proteolytic or microbial digests of natural proteins such as meats, fish, and milk (1, 3, 4, 7, 8, 13). The author speculated that many dipeptides digested easily by the hydrolysis of natural proteins could not be remaining as the dipeptides, and these dipeptides had not been evaluated

of their functions so far. Therefore, the author selected seven amino acids which were easily released by the hydrolysis of proteins or peptides (22-24) and synthesized dipeptides that contain these amino acids by Lal. Since toxic substances did not contained in the reaction mixtures of Lal, the reaction mixtures were evaluated directly in the screening without purification of dipeptide.

The amounts of dipeptides were not measured in this screening system in spite of different amounts of dipeptides contained in each reaction mixtures. This screening system was able to save us time and effort, and many reaction mixtures were able to be evaluated efficiently. As a matter of course, this screening system was useful for finding out salt enhancing dipeptide. Eight kinds of reaction mixtures were selected with two-step screening system, and most of the dipeptides which were predicted to be synthesized in those reaction mixtures were known as salt taste enhancers such as Leu-Ser (Koike M, Japanese patent JP2012-165740, 2012) and Arg-Lys (7). These results indicates that this screening system is able to select salt taste enhance dipeptides properly. Finally, the author found Met-Gly and Pro-Gly as the candidates for new salt taste enhancing dipeptides in this system. When Pro and Gly were used as substrates, Pro-Gly was synthesized almost exclusively form substrate specificity of TabS. On the other hand, the amount of Met-Gly was smaller than that of Met-Met in the reaction mixture using TabS, when Met and Gly were used as substrates; nevertheless Met-Gly was able to be detected as a candidate for new salt taste enhancing dipeptide in the reaction mixture. This result indicates that salt taste enhancing dipeptides were selected even there are several dipeptides synthesized in the reaction mixture. In addition, most reaction mixtures that were not selected with the second screening contained Arg. Arg is not salty itself, but enhances salt taste in NaCl solution (25). The author interpreted that salt taste intensities of reaction mixture containing Arg that was not selected with the second screening were affected by taste of Arg, not by taste of dipeptides.

The study of this chapter was designed to use reaction mixtures of Lal in

screening of salt taste enhancing. The author considers that this screening method described in this chapter is applicable for other taste evaluation of dipeptides.

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Chapter 3

Evaluations of Dipeptides as Salt Taste Enhancer Using a Sensory Assessment and a Taste Sensor

3.1. Introduction

In chapter 2, L-methionylglycine (Met-Gly) and L-prolylglycine (Pro-Gly) were found out as the candidates for salt taste enhancers using L-amino acid ligase (Lal). To confirm that effect, these dipeptides were evaluated by sensory assessment and objective taste assessment using authentic Met-Gly and Pro-Gly in this chapter.

The need for objective taste assessment has been increasing in addition to sensory test in recent years (1). The assays using taste receptors and taste sensor are mentioned as examples of objective taste assessment. Five basic tastes, sweet, umami, bitter, sour, and salty are mediated by each taste receptors in taste buds on tongue (2). Taste assays on the basis of taste receptors were constructed and screening of the taste-improvement agents was conducted (3). For instance, to identify molecules which affect the tastes, receptor-based assays have been reported. Servant et al. showed that SE-1, SE-2, and SE-3 (Fig. 1.1) were sweet enhancers through screening using a cell-based assay for the human sweet taste receptor (T1R2 and T1R3) (4). They also reported that these molecules had not sweet taste themselves. Sakurai et al. used human bitter taste receptor (hTAS2R16) in order to evaluate of the bitter-masking dipeptides such as L-glutaminyl-L-glutamic acid (Glu-Glu) and L-aspartyl-L-aspartic

acid (5). Glu-Glu was contained in acidic fraction of fish hydrolysate and it showed bitter-masking activity for bitter substances (6). Kim et al. demonstrated the interaction between bitter and umami taste using hTAS2R16 (7). In contrast, salt taste receptor mechanism has not been completely explained yet, and salt taste enhancers have not been found out using salt taste receptor. The study of the mechanism has been gradually advanced. Oka et al. elucidated that low concentration of NaCl activates the epithelial sodium channel (ENaC) (8) and high concentration of NaCl activates the bitter taste and sour taste receptors in addition to ENaC (9). Furthermore, Lu et al. reported that S3969 (Fig. 1.2) is the activator of the human ENaC (10). On the other hand, taste sensor has also developed recently (1). Taste sensor is composed of several kinds of lipid/polymer membranes and information of taste substances converts into electric signal (11). Taste sensor analysis is able to grasp taste characteristics as human tongue. For instance, to compere apple juices quality, or predict drug's bitterness, taste sensor was used (12, 13). There are reports of taste-improvement agents evaluated assessed by taste sensor. Maniruzzaman et al. evaluated masking effect of hot melt extruded paracetamol formulations (14), and Ito et al. evaluated that of artificial sweeteners on the bitter taste of H_1-antihistamines (13).

In this chapter, taste sensor was used as objective taste assessment, and the author also showed screening method using Lal (in chapter 2) was reliable one through the assessments.

The contents in this chapter were summarized in the research paper (15).

3.2. Materials and Methods

3.2.1. Materials

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

3.2.2. Sensory assessment

First, 0.60% (w/v) NaCl solution and 0.60% (w/v) NaCl solution containing 0.1% (w/v) Met-Gly (Met-Gly salt solution), or 0.1% (w/v) Pro-Gly (Pro-Gly salt solution) were prepared. Professional panelists compared salt taste intensities between 0.60% (w/v) NaCl solution and Met-Gly salt solution and between Met-Gly salt solution and Pro-Gly salt solution. Next, six kinds of NaCl solutions, 0.55, 0.60, 0.65, 0.70, 0.75, and 0.80% (w/v), were prepared. Seven samples containing the six kinds of the standard NaCl solutions and Met-Gly salt solution or Pro-Gly salt solution were rearranged in order of salt intensities by panelists who were able to rearrange the standard NaCl solutions in order of salt intensities in blind, and the panelists determined the position of Met-Gly salt solution and Pro-Gly salt solution among the standard NaCl solutions.

3.2.3. Taste sensor analysis

The Astree II electric tongue (Alpha M.O.S, Toulouse, France) was used for taste sensor analysis. This system is composed of auto sampler and seven cross-selective liquid sensors sensitive ionic and neutral chemical compounds responsible for taste, SRS (sour taste), STS (salt taste), UMS (umami), SWS, BRS, GPS, and SPS. The sensor response of each sensor and Ag/AgCl reference electrode for samples was measured. Six kinds of NaCl solutions, 0.55, 0.60, 0.65, 0.70, 0.75, and 0.80% (w/v), and 0.6% (w/v) NaCl solutions containing Met-Gly, Pro-Gly, and L-leucyl-L-serine (Leu-Ser) (Koike M, Japanese patent JP2012-165740, 2012) were prepared. Leu-Ser

was used as known salt enhancement dipeptide. Measuring of 25 mL each sample was conducted three times. The data was analyzed by AlphaSoft V12.46. Principal component analysis was conducted using these data. Met-Gly and Leu-Ser adding to water were also analyzed. Furthermore, response values of the sensor which is correlated with concentrations of standard NaCl solutions converted into relative values, and NaCl concentrations (conversion values) of Met-Gly salt solution, Pro-Gly salt solution, and Leu-Ser salt solution were determined by standard curve using the relative values of standard NaCl solutions. Met-Gly adding to water was also analyzed to evaluate the feature of Met-Gly itself. At that time, five kinds of standard NaCl solution, 0.01, 0.05, 0.10, 0.15, and 0.20% (w/v), were prepared. The operation and analysis were conducted by Yoshida K and Ikehama K (Alpha M.O.S Japan K.K., Tokyo, Japan).

3.3. Results

3.3.1. Sensory assessment

First, Professional panelists compared salt taste intensities between the 0.6% (W/V) NaCl solution and Met-Gly salt solution and between Met-Gly salt solution and Pro-Gly salt solution. They judged that salt taste intensity of Met-Gly salt solution was stronger than that of the 0.6% (W/V) salt solution ($p < 1.0$) and salt taste intensity of Pro-Gly salt solution was stronger than that of Met-Gly salt solution ($p < 0.15$). Met-Gly salt solution tasted like pickles, and its taste was different from NaCl solution itself. Compared with Met-Gly, Pro-Gly tasted similar to NaCl solution. Next, six kinds of the standard NaCl solutions and Met-Gly salt solution or Pro-Gly salt solution were rearranged in order of salt intensities by panelists, and both dipeptide

solutions positioned between 0.60 and 0.65% (w/v) NaCl solution. Furthermore, panelists judged that 0.1% (w/v) Met-Gly or Pro-Gly adding to water did not have salt taste itself. These sensory evaluations indicated that Met-Gly and Pro-Gly were new salt taste enhancing dipeptides.

3.3.2. Taste sensor analysis

First, Principal component analysis of 0.6% (w/v) NaCl solutions containing Met-Gly and Leu-Ser was conducted using the Astree II (Fig. 3.1). Both dipeptides solutions positioned the different area from that of the standard salt solutions, and

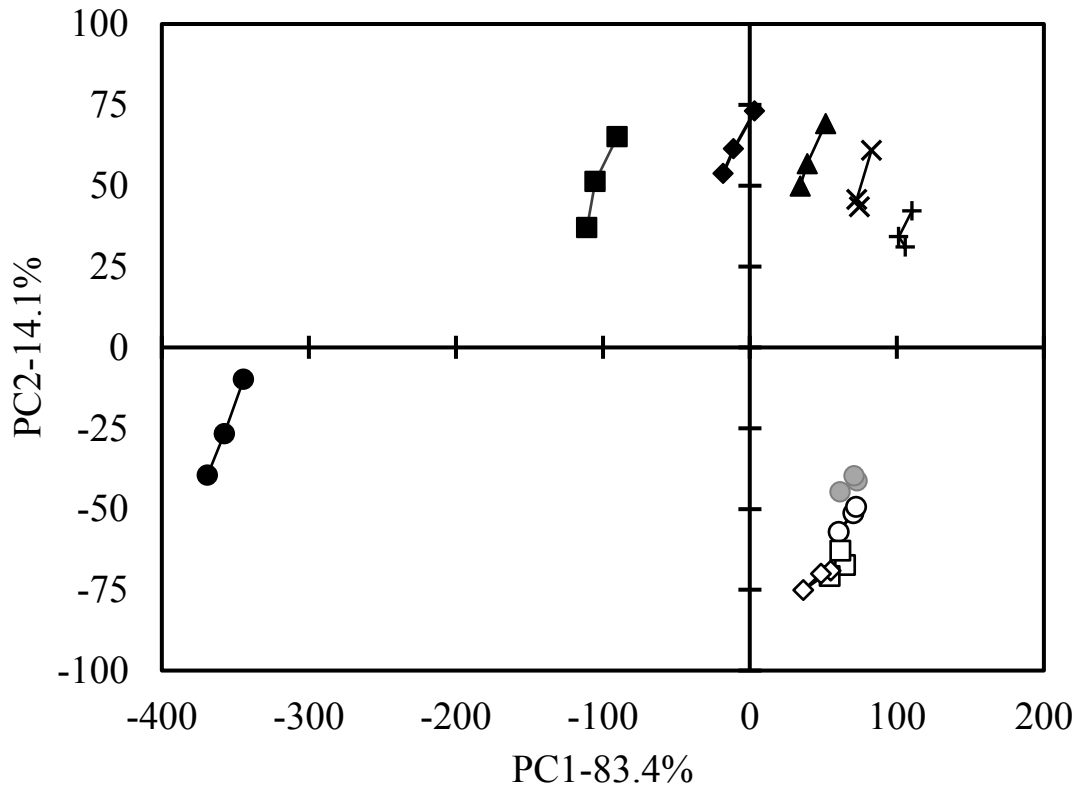

Fig. 3.1. Principal component analysis of 0.6% (w/v) NaCl solution containing Met-Gly and Leu-Ser.
The standard NaCl solutions showed 0.55 (●), 0.60 (■), 0.65 (◆), 0.70 (▲), 0.75 (×), and 0.80% (w/v) (+). 0.1% (w/v) Leu-Ser (●), 0.05 (○), 0.10 (□), and 0.20% (w/v) (◇) of Met-Gly were added to 0.60% (w/v) NaCl solution.

positioned at right area in a horizontal direction. On the other hand, Met-Gly and Leu-Ser adding to water positioned at left in a horizontal direction (Fig. 3.2). Next, response values of STS sensor that was correlated with concentrations of standard NaCl solutions converted into relative values, and standard curve was made using these relative values and the standard NaCl concentrations (Fig. 3.3.). NaCl concentrations of Met-Gly salt solutions and Leu-Ser salt solution were determined using this standard curve (Table 3.1.). All dipeptides salt solutions showed higher NaCl concentration than 0.60% (w/v). Furthermore, another standard curve was made using low concentration of standard NaCl solution (data not shown), and NaCl concentrations of 0.1% (w/v) Leu-Ser, 0.05, 0.10, and 0.20% (w/v) Met-Gly solutions not containing NaCl were under 0.01% (w/v).

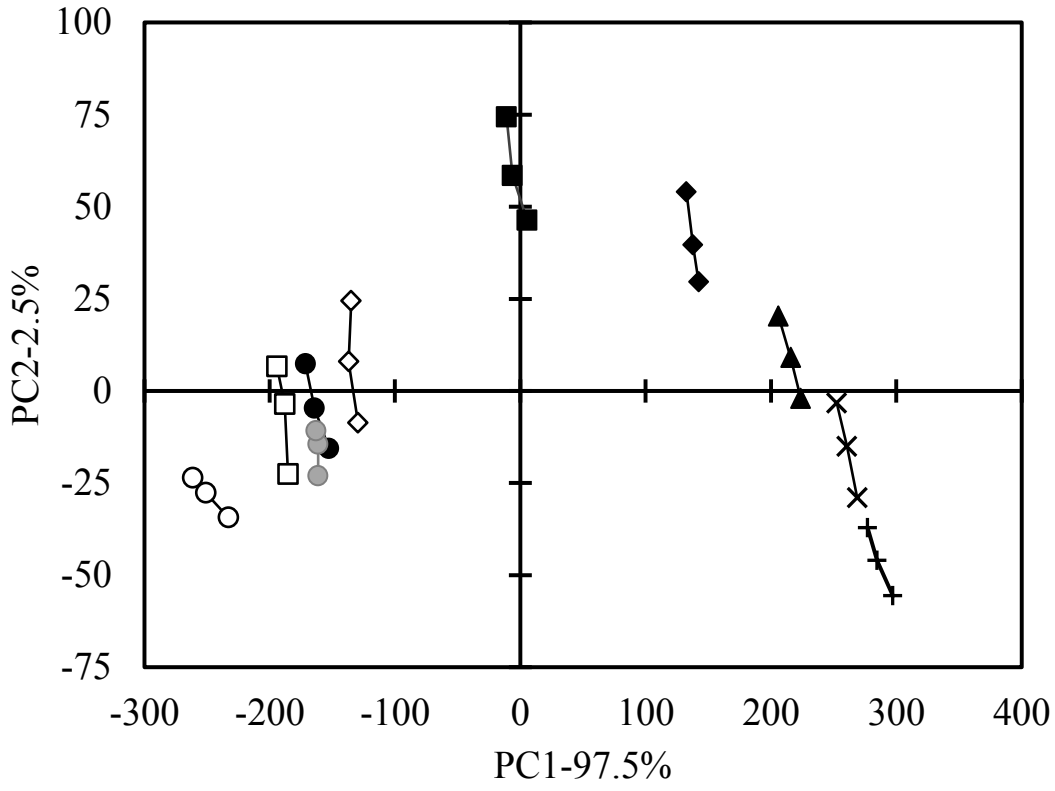

Fig. 3.2. Principal component analysis of water containing Met-Gly and Leu-Ser. The standard NaCl solutions showed 0 (●), 0.01 (■), 0.05 (◆), 0.10 (▲), 0.15 (×), and 0.20% (w/v) (+). 0.1% (w/v) Leu-Ser (●), 0.05 (○), 0.10 (□), and 0.20% (w/v) (◇) of Met-Gly.

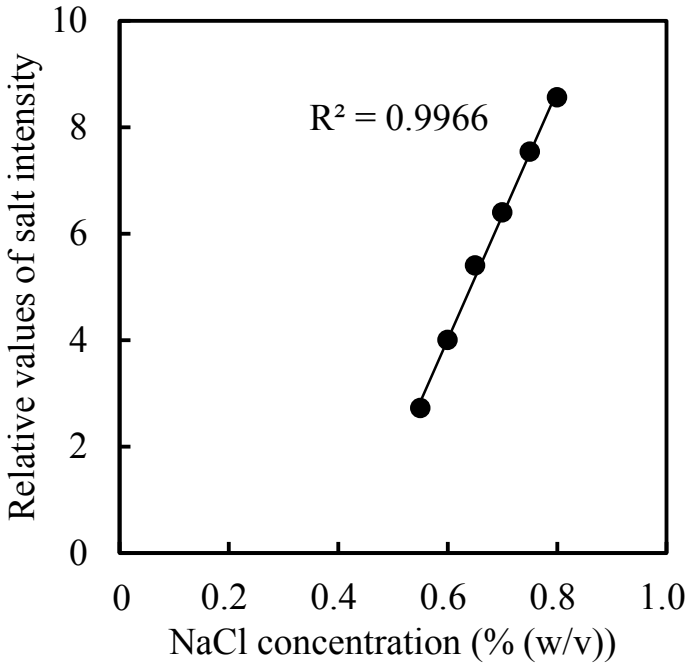

Fig. 3.3. The relationship between NaCl concentration and relative values of salt intensity measured by taste sensor.

Table 3.1. NaCl concentration of 0.6% (w/v) NaCl solution containing Leu-Ser and Met-Gly.

Dipeptide	Additive rate (% (w/v))	Relative value of salt intensity	NaCl concentration (% (w/v))
Leu-Ser	0.10	5.74	0.67
Met-Gly	0.05	5.86	0.68
	0.10	6.47	0.70
	0.20	7.32	0.74

NaCl concentration was calculated by the standard curve (Fig. 3.3.)

Pro-Gly was also analyzed by taste sensor, and NaCl concentration of Pro-Gly salt solutions was calculated. Because SRS sensor, not STS sensor, was correlated with concentration of standard NaCl solutions, the relative values of data from SRS sensor were used to make standard curve in this case. NaCl concentrations of 0.6% (w/v) NaCl solutions containing 0.05, 0.1, 0.2% (w/v) Pro-Gly were 0.63, 0.64, and 0.65% (w/v), respectively.

3.4. Discussion

In this chapter, the ability of Met-Gly and Pro-Gly as salt taste enhancers was confirmed by assessment using sensory assessment and taste sensor analysis. Met-Gly had been reported as whitening effect (16), and Pro-Gly had been reported as therapeutic agent (Sugihara, F. WO2012/102308 A1). However, both dipeptides had not been known as salt enhancers. This is the first report about effect of salt taste enhancement on Met-Gly and Pro-Gly. In the sensory assessment, two evaluations were conducted: (i) two-point discrimination test, comparing between NaCl solution and NaCl solutions containing Met-Gly (Met-Gly salt solution) or Pro-Gly (Pro-Gly salt solution) and (ii) rearrangement samples containing the standard NaCl solutions and Met-Gly salt solution or Pro-Gly salt solution in order of salt intensity. The evaluation method using the standard NaCl solutions similar to evaluation (ii) in this chapter was conducted by Schinder et al. (17). This evaluation was considered the reliable one though the NaCl concentration of sample was not determined strictly. Sensory assessment showed that salt intensity of Pro-Gly salt solution was stronger than that of Met-Gly salt solution from two-point discrimination test, and both dipeptide salt solutions positioned between 0.60 and 0.65% (w/v) NaCl solution from rearrangement evaluation. Panelists also confirmed that 0.1% (w/v) Met-Gly or

Pro-Gly adding to water did not have salt taste itself, and Kirimura et al. also had already reported that Pro-Gly have little taste (18). Therefore, Met-Gly and Pro-Gly were regarded not as salt replacers but as salt taste enhancement dipeptides by sensory evaluations.

On the other hand, two evaluations were also conducted using taste sensor: (i) grasping taste characteristics using principal component analysis with seven sensors and (ii) determination of NaCl concentration using one sensor that was correlated with concentrations of standard NaCl solutions. In this study, taste sensor analysis was conducted with focus on Met-Gly. When Met-Gly salt solution was analyzed with principal component analysis, it positioned different area from that of the standard NaCl solutions (Fig. 3.1). This indicated that Met-Gly salt solution had different taste from NaCl solution itself, and this result corresponded to the sensory evaluation as described above. Because the positions of the standard NaCl solutions moved to right in a horizontal direction depending on the NaCl concentrations, PC1 (horizontal axis) was showed the degree of salt intensity. NaCl solutions containing 0.10% (w/v) Leu-Ser, 0.05, 0.10, and 0.20% (w/v) Met-Gly positioned more right in a horizontal direction than 0.60% (w/v) NaCl solution, hence, salt intensity of these dipeptide salt solutions had stronger than that of 0.60% (w/v) NaCl solution. In contrast, though standard NaCl solutions with low concentration were identified individually and moved to right in a horizontal direction depending on the NaCl concentrations as well as Fig. 3.1., NaCl solutions containing 0.05, 0.10, and 0.20% (w/v) Met-Gly only gathered in one place around the area of water (Fig. 3.2). Therefore, it was judged that Met-Gly did not have salt taste itself, and this judgment also was supported by evaluation that NaCl concentration of 0.05, 0.10, and 0.20% (w/v) Met-Gly solutions not containing NaCl was under 0.1% (w/v) using standard curve. Though, in this

chapter, Met-Gly was analyzed using principal component analysis in detail, the author considers that Pro-Gly might exhibit the same behavior.

NaCl concentrations of NaCl solutions containing Leu-Ser, Met-Gly, or Pro-Gly were determined by standard curve using the relative values of the sensor that was correlated with concentration of standard NaCl solutions. All dipeptides salt solutions exhibited higher NaCl concentration than 0.60% (w/v), and these results corresponded to the sensory evaluation. Though the salt intensity of Pro-Gly salt solution was stronger than that of Met-Gly with taste assessment, the opposite result was obtained by taste sensor analysis. The author considers that further analysis will be needed.

When known salt enhancement dipeptides except for Leu-Ser were focused, some dipeptides exhibited the ability as salt taste enhancers by using other substances together. For instance, L-glutaminyl-L-threonine did not enhance salt taste itself, but showed the ability by using L-glutaminyl-L-threonine and bonito extract or L-arginine together, or using these three substrates at the same time (19). L-tryptophyl-L-tryptophan (Sakurai T, Tanaka M, Japanese patent JP2014-79213, 2014) exhibited the effect of salt taste enhancement stronger by using L-arginine hydrochloride together than using itself. The author regarded Met-Gly and Pro-Gly as useful salt taste enhancing dipeptides in comparison with these dipeptides, because Met-Gly and Pro-Gly showed the effect itself. It would be possible to enhance salt intensity of Met-Gly and Pro-Gly stronger by using other substances together. In addition, changing NaCl solution into other model materials such as instant processed food and noodle-soups will exhibit different salt intensity from Met-Gly and Pro-Gly salt solution because some salt taste enhancing dipeptides showed different salt intensity depending on the solutions (17).

Met-Gly and Pro-Gly were found out as salt taste enhancers in this chapter. Furthermore, new screening method using Lal constructed in chapter 2 was verified that desired dipeptides were selected properly from the results of this chapter.

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Chapter 4

Alteration of the Substrate Specificity of L-Amino Acid Ligase and Selective Synthesis of L-Methionylglycine as a Salt Taste Enhancer

4.1. Introduction

Studies of L-amino acid ligases (Lals) have focused on finding new Lals, which have thus far been identified in YwfE (1), BL00235 (2), TabS (3), and so others. In addition, determination crystal structures and alteration of the substrate specificities have been recently increased, but there was no report describing that alteration of substrate specificity of Lals leads to synthesize dipeptides selectively as planned. In this chapter, the author focused on BL00235 from *Bacillus licheniformis* NBRC 14081 to synthesize L-methionylglycine (Met-Gly) as s salt taste enhancement dipeptide (4) (chapter 3). BL00235 accepts only L-methionine (Met) and L-leucine (Leu) as the N-terminal substrates and prefers small residues such as L-alanine (Ala) and L-serine (Ser) as the C-terminal substrate (2). BL00235, possessing the unique substrate specificity characteristics, was expected to synthesize Met-Gly efficiently. In fact, BL00235 synthesized Met-Gly as a major product and L-methionyl-L-methionine (Met-Met) as a by-product, when Met and glycine (Gly) were used as substrates. If the Met-Gly is to be supplied by Lal efficiently, it is necessary to synthesize Met-Gly

selectivity without by-product. Therefore, the author decided to alter the substrate specificity of BL00235 based on its structure (PDB ID: 3VOT) (5). Alignment of amino acid sequences of five Lals (1, 2, 3, 6, 7) are shown in Fig. 4.1. Glu84 residue of BL00235 is the one of the few amino acid residues conserved in Lals, and contributes to stabilize magnesium ion (5, 8). Shomura et al. reported that Asn108 Glu109, and Leu110 residues of YwfE might affect the C-terminal substrates preferences based on its structure (PDB ID: 3VMM) (9). Asn108 and Leu110 residues of YwfE correspond to Phe83 and Pro85 residues of BL00235 (Fig. 4.1). As shown in Fig. 4.2., Phe83 and Pro85 residues make the space around C-terminal substrate narrow. BL00235 prefers

BL00235	75	P	F	D	G	V	M	T	L	F	E	P	A	L	P	88
YwfE	100	A	V	D	A	I	T	T	N	N	E	L	F	I	A	113
TabS	75	H	P	A	A	V	L	P	G	T	E	S	G	V	I	88
RSp1486a	75	G	P	D	A	I	F	T	F	S	E	F	L	L	K	88
RizA	73	P	F	D	H	I	V	S	T	T	E	K	S	I	L	86

BL00235	89	F	T	A	K	A	A	E	A	L	N	L	P	G	L	102
YwfE	114	P	M	A	K	A	C	E	R	L	G	L	R	G	A	127
TabS	89	V	A	D	L	L	A	A	A	L	Q	L	P	G	N	102
RSp1486a	89	S	V	S	E	L	A	A	E	F	G	L	R	A	V	102
RizA	87	T	G	G	F	L	R	S	Y	F	G	I	A	G	P	100

Fig. 4.1. Alignment of primary structures of Lals.
Amino acid conserved among the five sequences of Lals in white with black shading.

Gly, Ala, and Ser, which have slim side chains, as C-terminal substrates because of the narrow space around C-terminal substrate (5). The author deduced that Met might not be recognized as a C-terminal substrate if the space around C-terminal substrate is occupied by other amino acid. In this chapter, according to this hypothesis, the author focused on the Pro85 residue, of which side chain is smaller than that of Phe83, and Pro85 was replaced with L-phenylalanine (Phe), L-tyrosine (Tyr), and L-tryptophan

(Trp) having bulky aromatic side chains by site-directed mutagenesis. These mutants lost the capacity to synthesize Met-Met, during the synthesis of Met-Gly. The results shown in this chapter demonstrate a new and useful application for Lal.

The contents in this chapter were summarized in the research paper (10). Amino acids are written in three letter codes as shown in chapter 2.

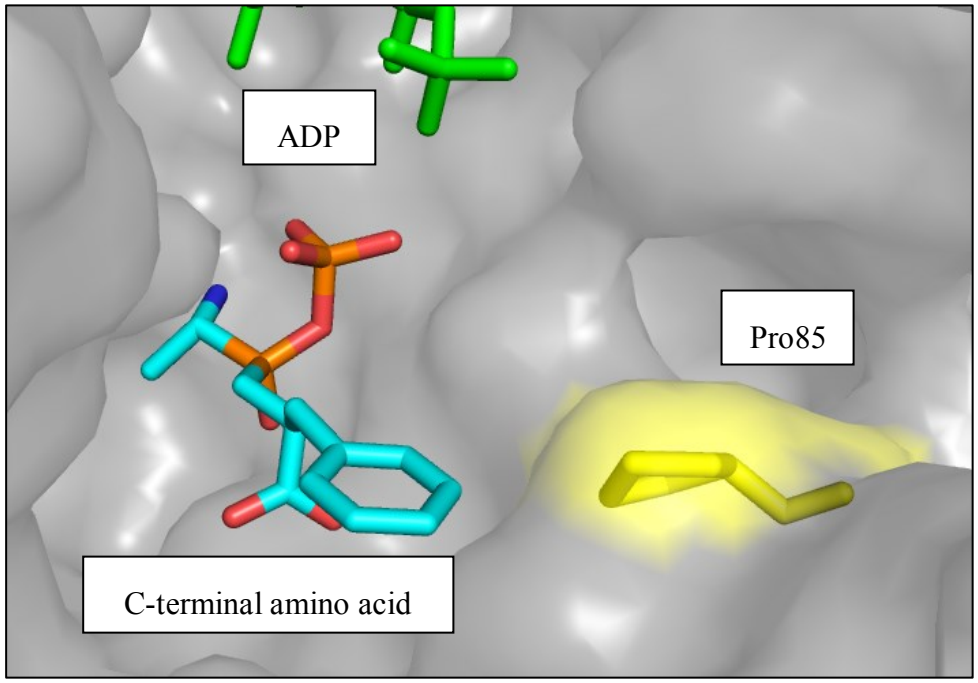


Fig. 4.2. Structure of YwfE in complex with the phosphinate L-alanyl-L-phenylalanine analog (P-analog) is superimposed onto the substrate recognition site of BL00235.
ADP (green), the P-analog (C atoms in light blue) and Pro85 residues (blue) were drawn with stick models. The figure was prepared using PyMol.

4.2. Materials and Methods

4.2.1. Materials

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

4.2.2. Site-directed mutagenesis

Site-directed mutagenesis was conducted using 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), and the primers listed in Table 4.1. The generation of the desired mutations was confirmed through DNA sequencing.

Table 4.1. Oligonucleotides used to generate BL00235 mutants.

Mutation	Oligonucleotide (5' to 3')[a]
P85F	ttt gaa TTC gct ttg cct ttc acg gca aaa gct
	caa agc GAA ttc aaa cag tgt cat cac gcc gtc
P85Y	ttt gaa TAC gct ttg cct ttc acg gca aaa gct
	caa agc GTA ttc aaa cag tgt cat cac gcc gtc
P85W	ttt gaa TGG gct ttg cct ttc acg gca aaa gct
	caa agc CCA ttc aaa cag tgt cat cac gcc gtc
P85G	ttt gaa GGG gct ttg cct ttc acg gca aaa gct
	caa agc CCC ttc aaa cag tgt cat cac gcc gtc

[a] Mutated nucleotides are capitalized.

4.2.3. Enzyme preparation

Method for preparation of TabS was described in chapter 2. The genes encoding BL00235 and mutants were previously cloned into the pET21a(+) vectors (2). Recombinant *Escherichia coli* BL21 (DE3) cells were cultivated in 3 mL LB medium (1% bacto tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 50 μg/mL ampicillin at 37°C for 5 h with shaking at 160 rpm. For the main culture, 150 mL LB medium containing 50 μg/mL ampicillin was inoculated with 1.5 mL preculture broth,

and cultivated with shaking on a gyratory shaker (120 rpm) at 37°C. After cultivating for 2 h, 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to the medium, and cultivation conducted at 25°C for an additional 19 h with shaking on a gyratory shaker (120 rpm). The cells were collected with centrifugation (3,000 × g, 10 min, 4°C) and washed twice with 100 mM Tris-HCl buffer (pH 8.0). After washing, the cells were suspended in 100 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0) and then lysed by sonication at 4°C. The lysate was centrifuged (20,000 × g, 30 min, 4°C), and the supernatant was purified and fractionated with a His Gravitrap™ affinity column (GE Healthcare, Buckinghamshire, UK). 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).

4.2.4. Dipeptide synthesis using purified enzyme

The standard reaction mixtures (300 μL) contained 20 mM Met and 20 mM Gly, 40 mM Met, 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 BL00235 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 enzymes were removed by centrifugation (20,000 × g, 20 min, 4°C). To compare the amount of phosphate produced between in the reaction mixtures of the wilt-type BL00235 and the mutant, Met was reacted with 20 proteogenic amino acids under standard conditions. To evaluate the relationship between the amount of Met-Gly synthesized and the concentration of the wild-type BL00235 and the mutant, the reactions were performed for 60 min with various concentration of each enzyme (0.1-0.5 mg/mL) under standard conditions. To determine the kinetic parameters, the reactions were performed with various concentration of Met (5-100 mM), Gly (2-60

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.

4.2.5. Analysis

The amounts of phosphate produced in reaction mixtures were measured with a Determiner L IP kit (Kyowa Medex, Tokyo, Japan) as the indicator of dipeptide synthesis. The amounts of dipeptides were analyzed by HPLC (L-2000 series; Hitachi High Technologies, Tokyo, Japan). The details of the analytical procedure were described previously (11). Data obtained by HPLC analyses were averages from three independent experiments, and error bars indicated standard deviation of the means.

4.3. Results

4.3.1. Synthesis of Met-Gly with TabS and BL00235

TabS was able to synthesize Met-Gly, but the amount of Met-Gly was smaller than that of Met-Met (4) (chapter 2). On the other hand, BL00235 has a unique substrate specificity of permission for Met and Leu only as the N-terminal substrates (2). The amounts of Met-Gly and Met-Met were compared using TabS and BL00235 (Fig. 4.3). They produced opposite ratios of Met-Gly to Met-Met, and BL00235 synthesized Met-Gly as a major product. In addition, neither TabS nor BL00235 could synthesize Gly-Gly and Gly-Met. Therefore, BL00235 was a suitable Lal for Met-Gly synthesis.

4.3.2. Site-directed mutagenesis of BL00235

Four mutants were constructed, replacing the Pro85 residue of BL00235 with Phe

(P85F), Tyr (P85Y), and Trp (P85W) which have bulky aromatic side chains, and with Gly (P85G) which has a small side chain. The expression and purification of the wild-type BL00235 and mutants were carried out, and the reaction was performed using 20 mM Met and Gly or 40 mM Met as substrates (Fig. 4.4). Neither Gly-Gly nor Gly-Met were shown in this figure because they were not detected. The P85F, P85Y, and P85W mutants lost the capacity of synthesizing Met-Met, but these mutants synthesized Met-Gly from Met and Gly (Fig. 4.4 (A)). Furthermore, these mutants did not synthesize Met-Met, even when Met was used as a substrate. By contrast, the wild-type BL00235 and the P85G mutant showed similar substrate specificities. These results indicated that the P85F, P85Y, and P85W mutants synthesized Met-Gly selectively.

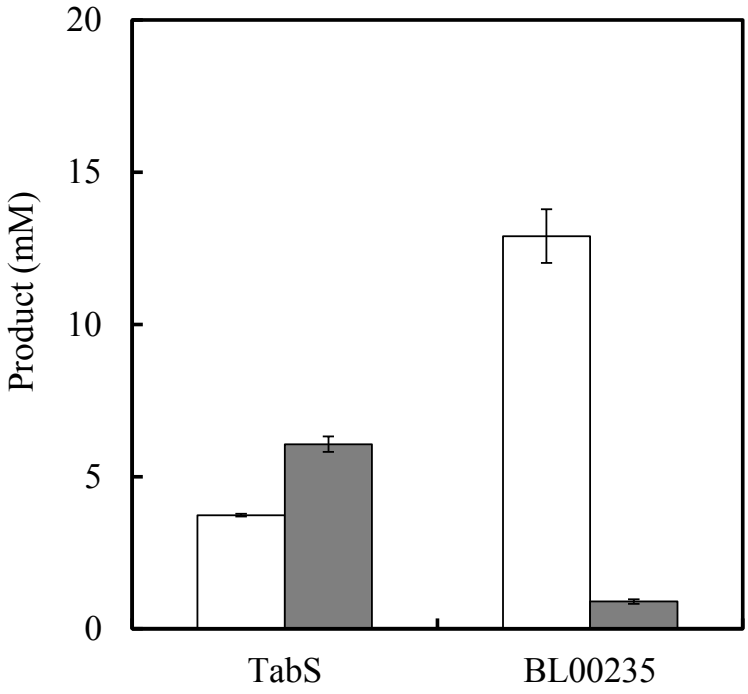


Fig. 4.3. Synthesis of Met-Gly (white bars) and Met-Met (dark bars) by TabS and BL00235.
Reaction mixture contained 20 mM Met and 20 mM Gly, 20 mM ATP, 20 mM $MgSO_4 \cdot 7H_2O$, and 0.5 mg/mL purified TabS or BL00235 in 50 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0). The reaction was performed at 30°C for 20 h.

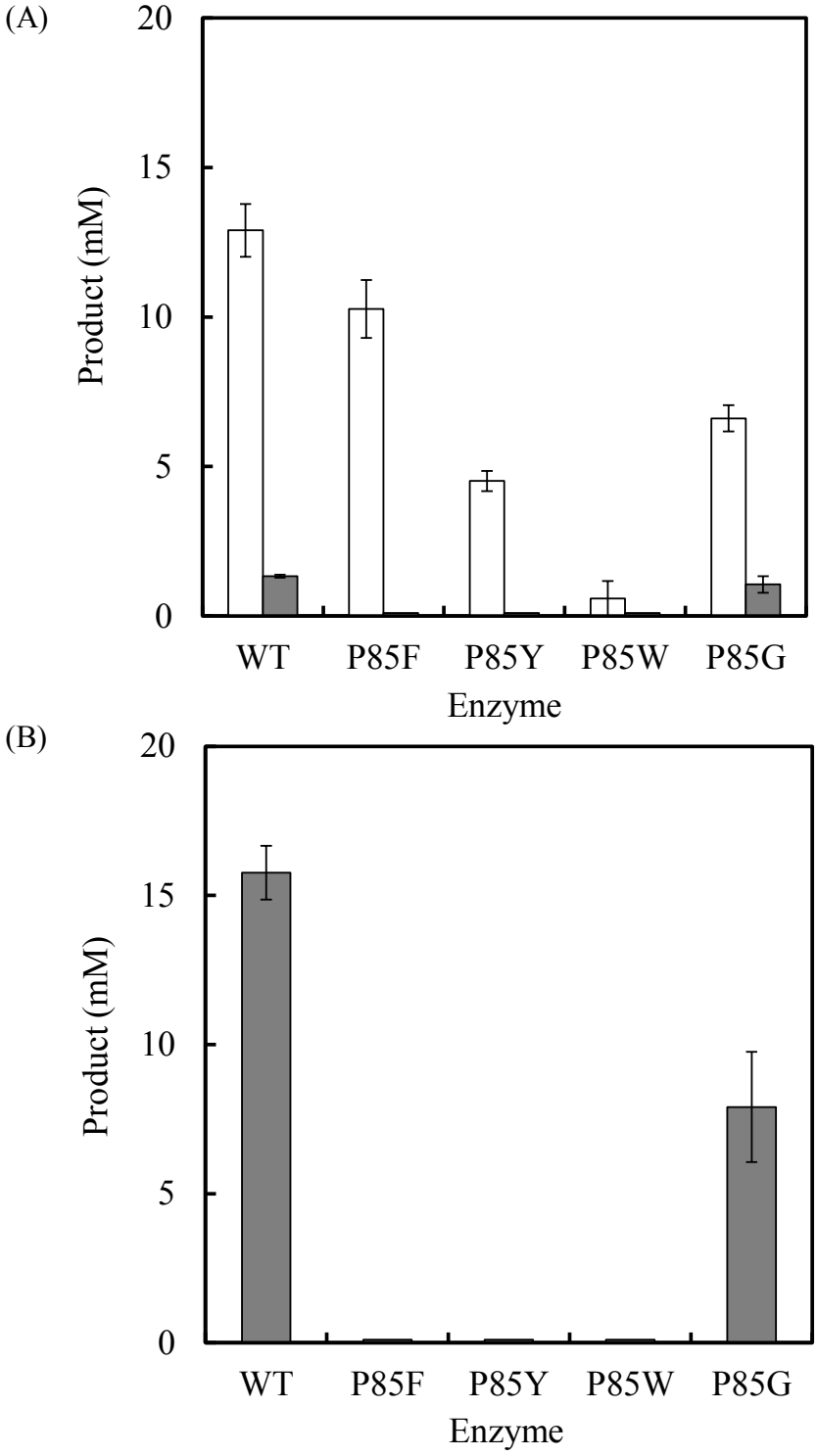


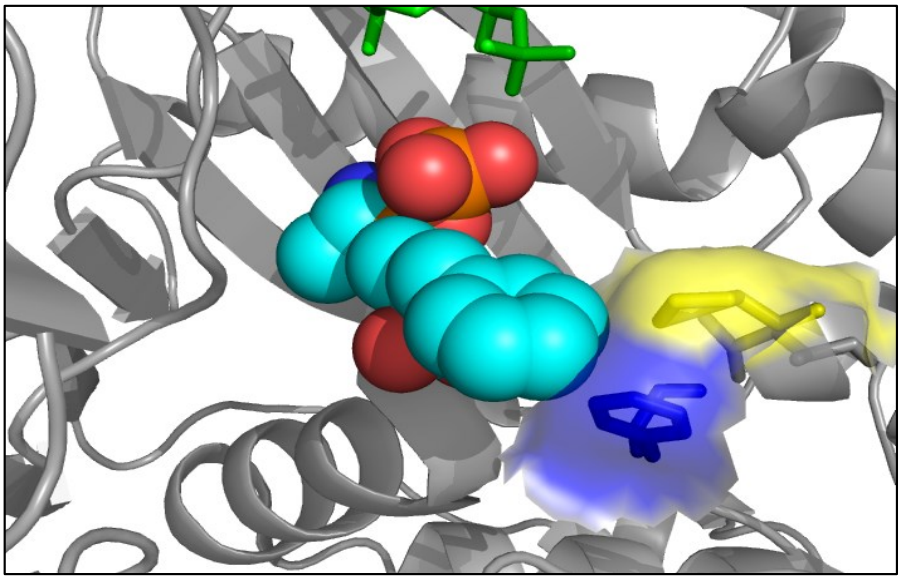
Fig. 4.4. Synthesis of Met-Gly (white bars) and Met-Met (dark bars) by the wild-type BL00235 and mutants.

Reaction mixture contained 20 mM Met and 20 mM Gly (A), or 40 mM Met (B), 20 mM ATP, 20 mM $MgSO_4 \cdot 7H_2O$, and 0.5 mg/mL purified TabS or BL00235 in 50 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0). The reaction was performed at 30°C for 20 h.

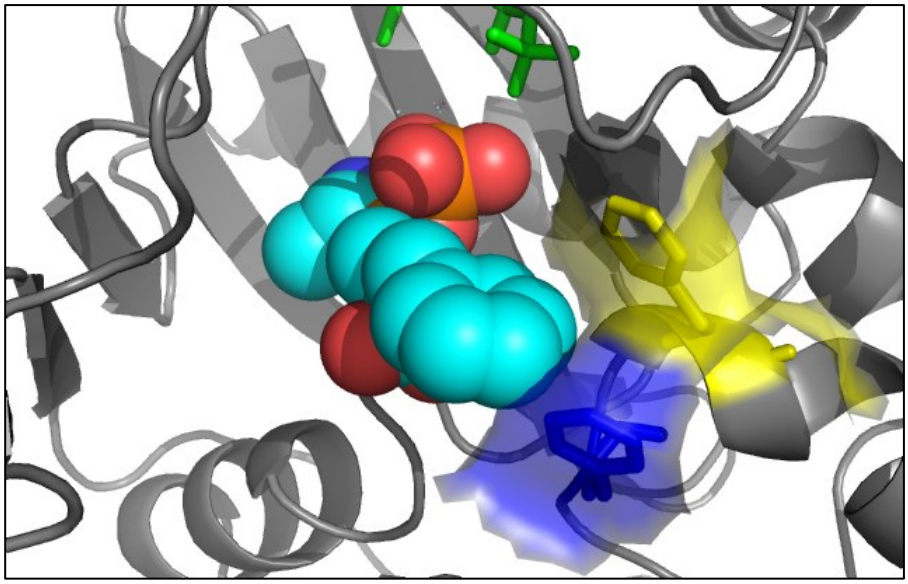
4.3.3. Predicted structure of the BL00235 mutants

The P85F mutant, the P85Y mutant, and P85W mutant lost the capacity of synthesizing Met-Met (Fig. 4.4), which indicated the binding pocket of these mutants was smaller than that of the wild-type BL00235. The structures of these mutants were predicted by SWISS-MODEL (Fig. 4.5).

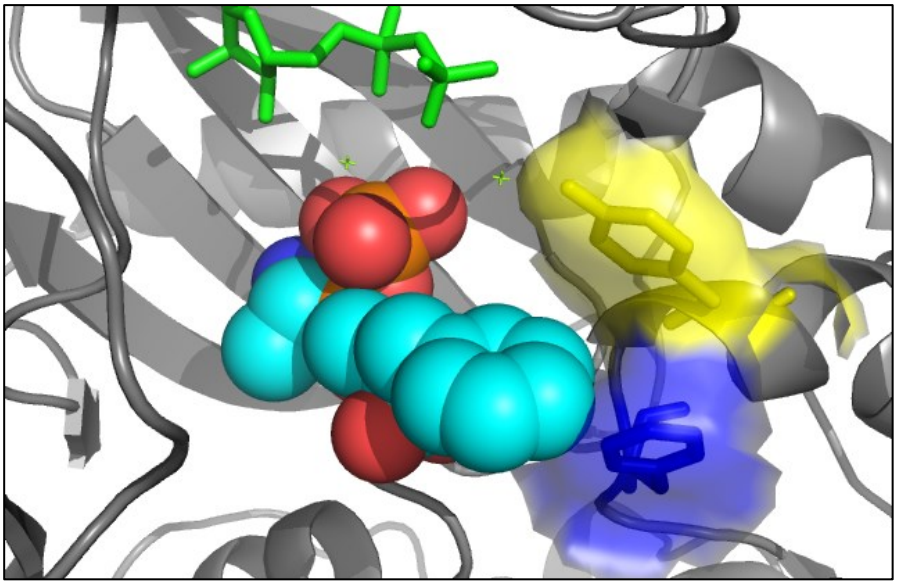
(A)



(B)



(C)



(D)

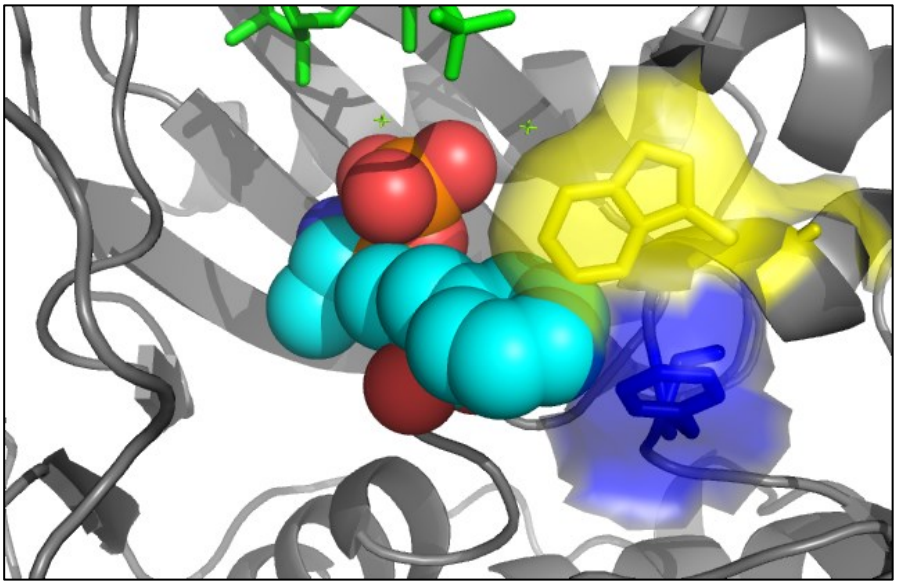


Fig. 4.5. The recognition site of the wild-type BL00235 (A), the P85F mutant (B), the P85Y mutant (C), and the P85W mutant (D). The structures of the mutants were predicted by SWISS-MODEL.

ADP (green), Phe83 (blue), and Xaa85 (yellow) residues were drawn with stick models. The P-analog (C atoms in light blue) were drawn with spheres model. The figures were prepared using PyMol.

The proper structures of the mutants were not known. However, these predicted structures in Fig. 4.5 showed that the side chains of Phe, Tyr, and Trp residues of the mutants were larger than Pro residue of wild-type BL00235 and occupied the space around C-terminal substrates. The P85F mutant was used for further analysis because it synthesized more than twice the amount of Met-Gly as the P85Y mutant did and was considered more useful for Met-Gly synthesis..

4.3.4. Characterization of the P85F mutant

The P85F mutant synthesized Met-Gly selectively. The author predicted that C-terminal substrates of the P85F mutant might be altered. Therefore, Met was reacted with 20 proteogenic amino acids to compare the amount of phosphate produced

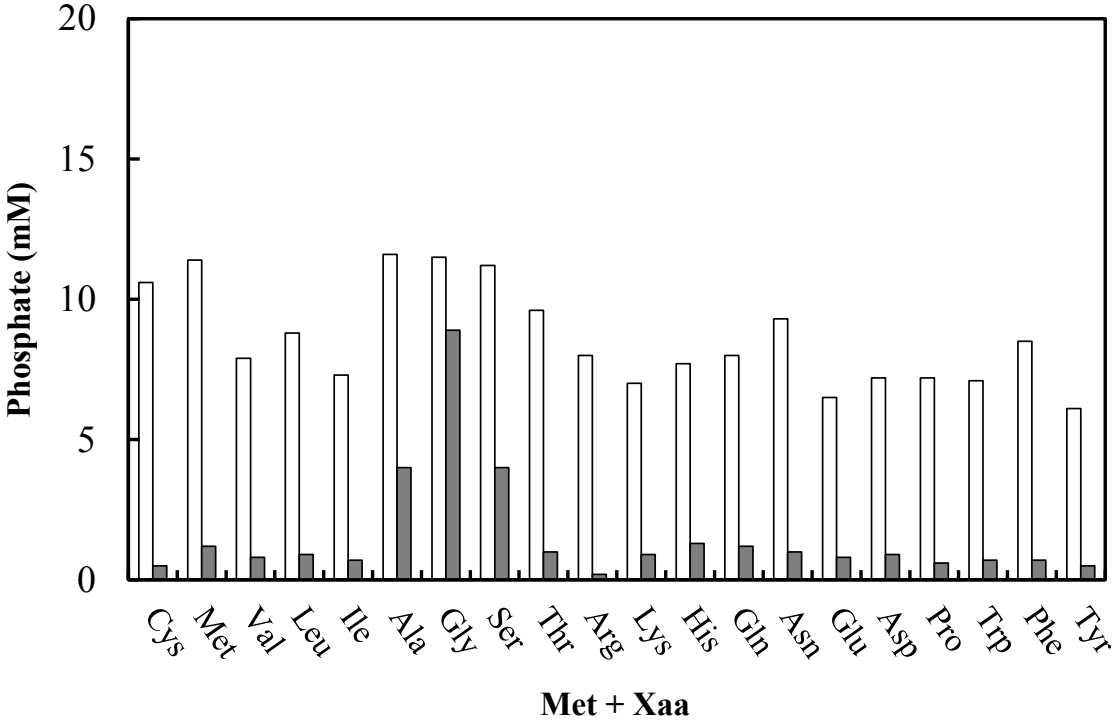


Fig. 4.6. Comparison of the amount of phosphate produced in the peptide synthesis between the wild-type BL00235 (white bar) and the P85F mutant (gray bar).

Reaction mixture contained 20 mM Met and 20 mM Xaa, 20 mM ATP, 20 mM $MgSO_4 \cdot 7H_2O$, and 0.5 mg/mL purified TabS or BL00235 in 50 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0). The reaction was performed at 30°C for 20 h.

between in the reaction mixtures of the wilt-type BL00235 and the P85F mutant (Fig. 4.6). The all reaction mixtures of the wild-type BL00235 contained over 5 mM phosphate. By contrast, there was little phosphate in the reaction mixtures of the P85F mutant except for the combinations of Met and Ala, Met and Gly, and Met and Ser as substrates. This large difference in the amounts of released phosphate between both enzymes indicated that the P85F mutant had affinity for only Ala, Gly, and Ser which had small side chains and lost the capacity of synthesizing Met-Met, although the amounts of released phosphate were not exactly the same as that of synthesized dipeptides.

Time course of Met-Gly synthesis was examined using the wild-type BL00235 and the P85F mutant (Fig. 4.7). The amount of Met-Gly synthesized by the P85F mutant

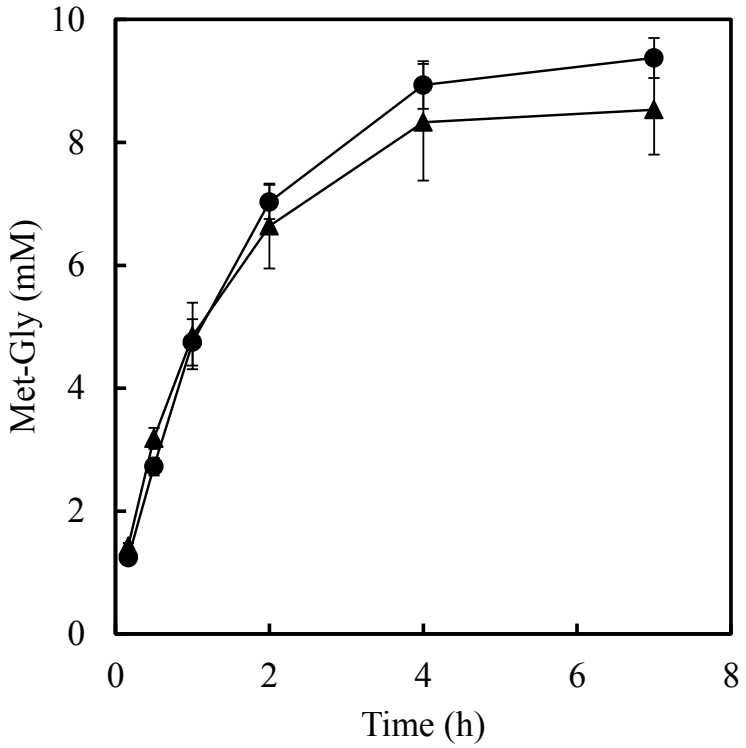


Fig. 4.7. Time course of Met-Gly synthesis by the wild-type BL00235 (●) or the P85F mutant (▲).
Reaction mixtures contained 20 mM Met and 20 mM Gly as substrates. The reaction was performed at 30°C

appeared to be slightly higher at the initial stage of reaction, but gradually became lower than that synthesized by the wild-type BL00235. In addition, the linear relationship was obtained between concentration of the wild-type BL00235 or the P85F mutant and the amount of Met-Gly synthesized by the wild-type BL00235 and the P85F mutant (Fig. 4.8).

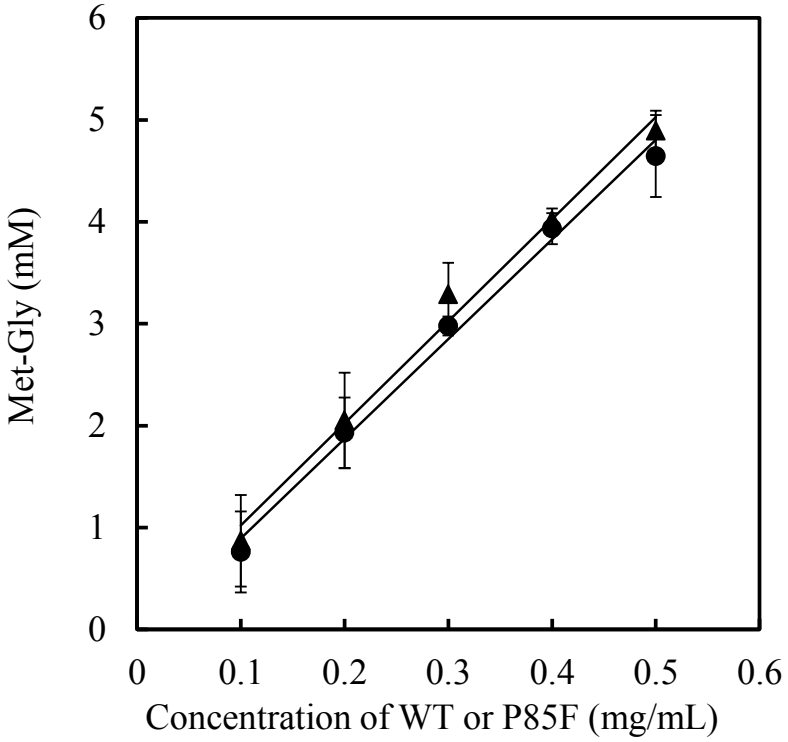


Fig. 4.8. Synthesis of Met-Gly using various concentration of the wild-type BL00235 (●) or the P85F mutant (▲).
Reaction mixture contained 20 mM Met and 20 mM Gly as substrates. The reaction was performed at 30°C for 60 min.

The kinetic parameters of Met-Gly synthesis were determined. According to Hanes-Woolf linearization (Fig. 4.9), the K_ms values and the V_{max} values for Met-Gly synthesis of the wild type BL00235 and the P85F mutant were shown in Table 4.2.

Finally, the relationship was examined between the concentration of Met or Gly and the amount of Met-Gly synthesized by the P85F mutant (Fig. 4.10) and

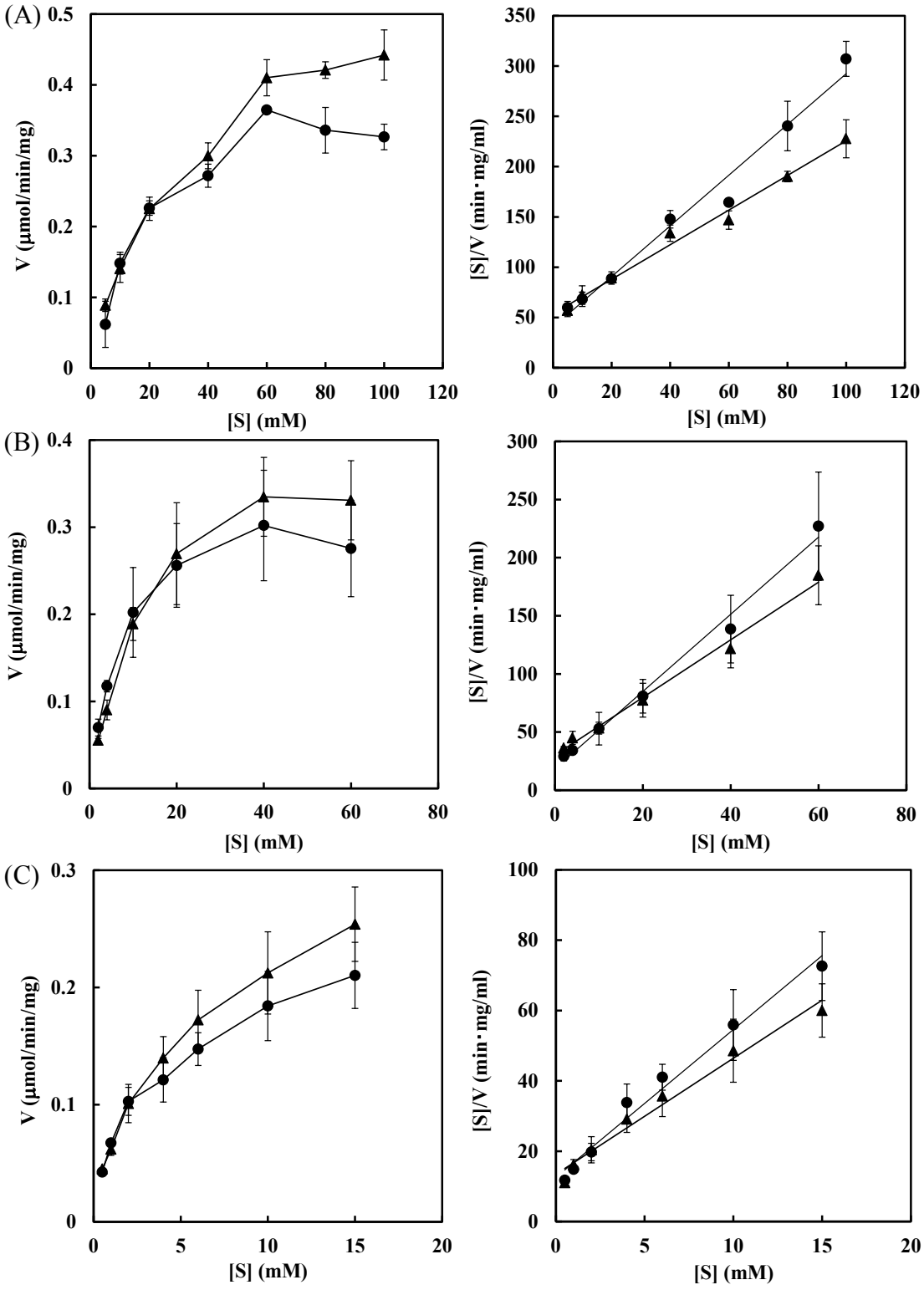


Fig. 4.9. Kinetic analysis of Met-Gly-synthesizing reaction by the wild-type BL00235 (●) and the P85F mutant (▲).

Plots of reaction velocity (V) vs. substrate concentration ([S]) curves (left shapes), and [S]/V vs. [S] curves (Hanes-Woolf plot; right). The reactions were performed by varying the concentration of Met (A), Gly (B), or ATP (C) at 30°C for 60 min.

the relationship between the concentration of Met or Gly and the amount of Met-Gly and Met-Met synthesized by the wild-type BL00235 (Fig. 4.11). The amount of Met-Gly synthesized by the P85F mutant was increased as Met concentration increased, whereas it was barely affected by changing in Gly concentration. The amount of Met-Met was not shown in Fig. 4.10, because Met-Met was not detected in any of the reaction mixture. On the contrary, the amount of Met-Met synthesized by the wild-type BL00235 was increased with an increase in Met concentration (Fig. 4.11).

Table 4.2. Kinetic parameters of Met-Gly-synthesizing reaction by the wild-type BL00235 and the P85F mutant.

	K_m (mM)			V_{max}
	Met	Gly	ATP	(nmol·min^{-1}·mg protein^{-1})
WT	17.1±0.962	3.91±0.371	3.07±0.435	407±40.8
P85F	31.4±4.84	10.3±0.842	3.95±0.286	580±40.1

The reaction was performed by varying the concentration of Met, Gly, and ATP.

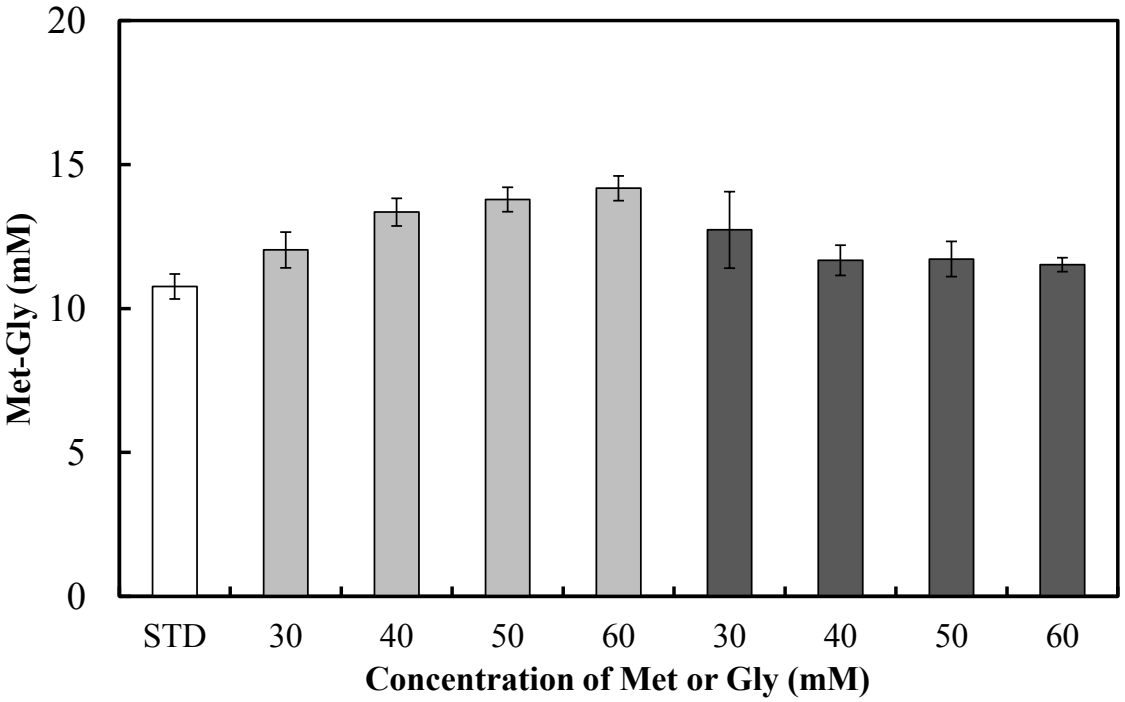


Fig. 4.10. Effect of substrate concentration on Met-Gly synthesis by the P85F mutant.

Reaction mixtures contained 20-60 mM Met and 20 mM Gly (STD, white bar), and 20 mM Met and 30-60 mM Gly (dark gray bars) as substrates.

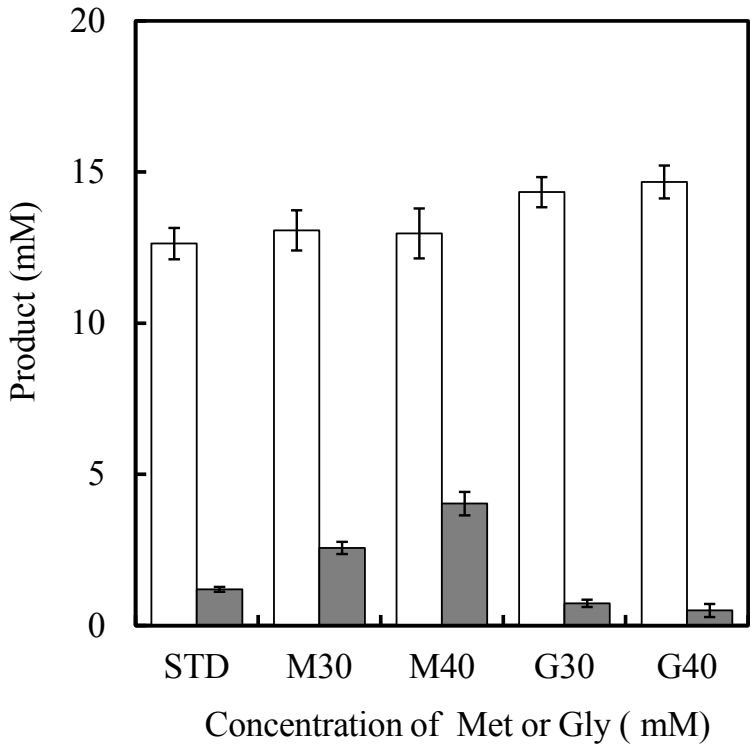


Fig. 4.11. Effect of substrate concentration on Met-Gly (white bars) and Met-Met (dark bars) synthesis by the wild-type BL00235.
Reaction mixture contained 20 mM Met and Gly (STD), 30 mM Met and 20 mM Gly (M30), 40 mM Met and 20 mM Gly (M40), 20 mM Met and 30 mM Gly (G30), or 20 mM Met and 40 mM Gly (G40) as substrates.

4.4. Discussion

In this chapter, the author constructed the mutants of BL00235 and succeeded in obtaining the mutants, such as P85F and P85Y, synthesized salt taste enhancing dipeptide, Met-Gly, selectively. Few reports on the alteration of Lals with structure-based site-directed mutagenesis have been published (8, 9). The objectives of mutant construction can be roughly divided into two types: (i) to reveal the catalytic function (8, 9) and (ii) to alter substrate specificity in order to synthesize useful dipeptides (8). This study had the latter one. Tsuda et al. indicated that single mutations of YwfE are able to alter the substrate specificity and suggested that alteration of substrate specificity of Lal might have led to synthesize desirable dipeptides (8).

However, no study has demonstrated that changing the substrate specificity of Lals by a single mutation made the mutants synthesize dipeptides as planned. Therefore, the study in this chapter is the first of the successful selective synthesis of a useful dipeptide using Lals by site-directed mutagenesis.

Arai. et al. previously reported phosphate analysis of reaction mixtures showed that TabS tends to choose Met as an N-terminal substrate and Gly as a C-terminal substrate (3). On the other hand, BL00235 has strict substrate specificity; it accepts only Met and Leu as C-terminal substrate and prefers small residues (2). The author hypothesized that both Lals synthesize Met-Gly as a major product, but experimental results showed that TabS synthesized Met-Met as major product (Fig. 4.3). These results show that the types of dipeptides synthesized by TabS changed according to the amino acids combination. Hence, BL00235 was selected, which synthesized Met-Gly as a major product more efficiently. In the reaction mixture of BL00235, there was a small amount of Met-Met. The structure of BL00235 suggests that the C-terminal substrate preference for amino acids with small residues is related to the Phe83 and Pro85 residues that positioned around C-terminal substrate (5). The author predicted that Met might not be recognized as a C-terminal substrate if the space around C-terminal substrate is occupied by other amino acid and that Met-Gly can be synthesized selectively without the synthesis of Met-Met. The author selected Phe, Tyr, and Trp, which have bulky aromatic side chains, to replace Pro85 residues.

Indeed, these mutants lost the capacity to synthesize Met-Met. Furthermore, the P85F and P85Y mutants maintained the capacity to synthesize Met-Gly (Fig. 4.4). The P85F mutant synthesized more than twice as much Met-Gly as the P85Y mutant. Interestingly, the amount of Met-Gly differed among the three mutants depending on the size of the side chain. The author considered that the side chain of Phe at the 85

position might be suitable for synthesizing Met-Gly selectively. On the contrary, Gly is not able to enter the pocket around C-terminal substrate of the P85Y mutant as easily as that of the P85F mutant because the Tyr side chain is slightly too large compared with Phe side chain. In addition, the P85W mutant, which has the largest side chain among Phe, Tyr and Trp, prevents even Gly as a C-terminal substrate from entering the binding pockets and loses most of its capacity to synthesize Met-Gly. The predicted structures of the P85F, P85Y, and P85W mutants also showed that the space around C-terminal substrate was occupied by Phe, Tyr and Trp residues of the mutants more than Pro residue of the wild-type BL00235, and the size of the space was dependent on that of the side chain. The wild-type BL00235 has the largest size of pocket around C-terminal substrate, followed in order of the P85F, the P85Y, and the P85W mutants (Fig. 4.5). Furthermore, phosphate analysis indicated that the substrate specificity of the P85F mutant was altered and the P85F mutant maintained affinity for only Ala, Gly, and Ser, which had small side chains (Fig. 4.6). These results also support the hypothesis that amino acid residues with bulky side chains at position 85 affect the C-terminal substrate specificity.

The increase of apparent K_ms values of the P85F mutant for Met, Gly suggested that the affinity with substrate for the P85F mutant was lower than that by the wild-type BL00235. Compared with that of the wild-type BL00235, the amount of Met-Gly synthesized by the P85F was lower. The author deduced that the difference of affinity with substrate leads to decrease the amount of Met-Gly. In contrast, the V_{max} value for Met-Gly synthesis by the P85F mutant was little higher than that by the wild-type BL00235. According to the time course of Met-Gly synthesis, the amount of Met-Gly synthesized by the P85F mutant was appear to be slightly higher than that by the wild-type BL00235 at the initial stage of reaction (Fig. 4.8). These characteristics

of the P85F mutant might affect the V_{max} value. When the amount of Met increased, the P85F mutant synthesized Met-Gly much more (Fig. 4.10). On the contrary, the amount of Met-Met synthesized by the wild-type BL00235 increased with an increase of Met. These results also support the assumption that Met is not recognized as a C-terminal substrate by the P85F mutant.

In this chapter, the author achieved the goal of selectively synthesizing the salt taste enhancing dipeptide Met-Gly. The application of Lals was able to be explored. The results in this chapter will contribute to the synthesis of other useful dipeptides and makes Lals easier to use than ever before.

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Chapter 5

Synthesis of L-Prolylglycine as a Salt Taste Enhancer by Site-Directed Mutagenesis of L-Amino Acid Ligase

5.1. Introduction

The mutant which synthesized L-methionylglycine (Met-Gly) selectively was obtained by altering the substrate specificity based on the crystal structure of BL00235 in chapter 4 (1, 2). This was the first report of succeeding in synthesizing useful dipeptide selectively using L-amino acid ligase (Lal) by site-directed mutagenesis. In chapter 4, the amino acid residue determining the C-terminal substrate specificity was focused on and the mutant that synthesized Met-Gly selectively was constructed by replacing Pro85 residue with phenylalanine (Phe) or Tyrosine (Tyr). The author deduced that the amino acid residue at position 85 had a key role in enzyme activity from these results. 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) (3). Therefore, these findings were applied to other Lal in this chapter.

In chapters 2 and 3, new salt taste enhancing dipeptides were searched, and L-prolylglycine (Pro-Gly) was found to have the effect in addition to Met-Gly. When

20 mM proline (Pro) and 20 mM glycine (Gly) were used as substrate, TabS (4), a Lal from *Pseudomonas syringae*, synthesized 8.3 mM Pro-Gly (chapter 2). TabS hardly synthesized few other three dipeptides, L-prolyl-L-proline (Pro-Pro), glycyl-L-proline (Gly-Pro) and glycylglycine (Gly-Gly), from its substrate specificity of preferring Pro and Gly as the N- and C-terminal substrate, respectively (4). It would be possible to increase the amount of Pro-Gly by replacing suitable amino acid residues of TabS with other amino acid residues. The amino acid residues, which positioned around N- and C-terminal substrate, were selected based on the results of BL00235 (Chapter 4) and the predicted structure of TabS. Alignment of amino acid sequences of five Lals (4-8) are shown in Fig. 5.1, and predicted structure of TabS is shown in Fig. 5.2. First, the author focused on Ser85 residues of TabS that corresponds to Pro85 residue of BL00235 and Leu110 residue of YwfE (Fig. 5.1) and positions around C-terminal

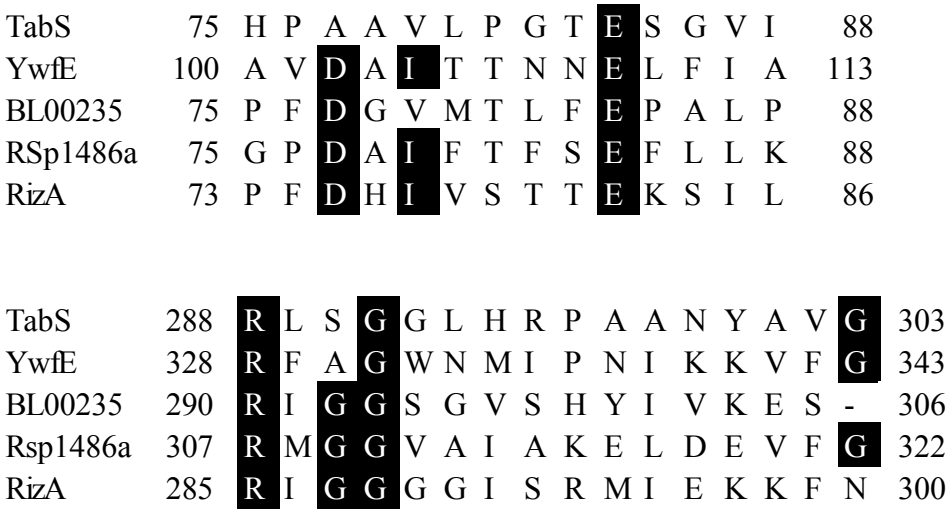

Fig. 5.1. Alignment of primary structures of Lals.
Amino acid conserved among the five sequences of Lals in white with black shading.

substrate (Fig. 5.2). Next, His294 residue that positions around N-terminal substrate was focused on (Fig. 5.2). This residue corresponds to Met334 residue of YwfE, which is one of the residues for determining the N-terminal substrate specificity (9). In this

chapter, Ser85 and His294 residues were replaced with 20 proteogenic amino acids, and Pro-Gly-synthesis reactions were conducted. The S85T and the H294D mutants synthesized more Pro-Gly than the wild-type TabS. Furthermore, the S85T/H294D double mutant synthesized considerably more Pro-Gly than the each single mutant. The author achieved demonstrating that the amino acid residue at position 85 affects enzyme activity in common between TabS and BL00235.

The contents in this chapter were summarized in the research paper (10). Amino acids are written in three letter codes, as shown in chapter 2

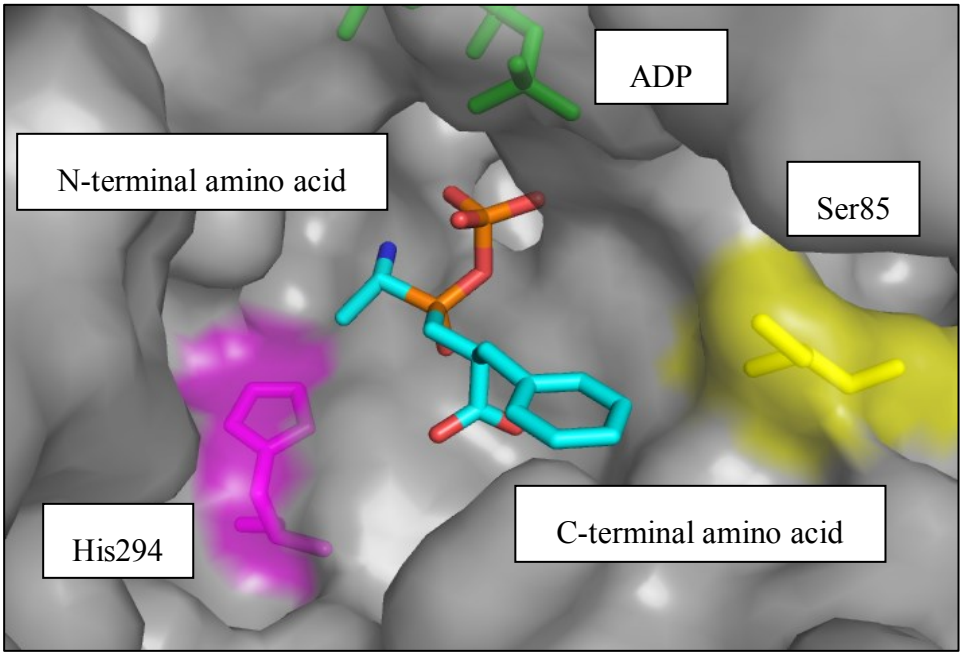

Fig. 5.2. The recognition site of TabS predicted by SWISS-MODEL.
ADP, the P-analog Ser85, and His294 residues were drawn with stick models. The figure was prepared using PyMol.

5.2. Materials and methods

5.2.1. Materials

All chemicals used in this study are commercially available and are of

chemically pure grade.

5.2.2. Site-directed mutagenesis

Site-directed mutagenesis was conducted using polymerase chain reaction with KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) based on the method on a QuikChange site-directed mutagenesis kit (Agilent Technologies, CA, USA), and the primers listed in Table 5.1. Ser85 and His294 were replaced with 20 proteogenic amino acids. The primers not listed in Table 5.1 had already constructed in the author's laboratory. The generation of the desired mutations was confirmed through DNA sequencing.

5.2.3. Enzyme preparation

Preparations of TabS and the mutants were described in chapter 2.

5.2.4. 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 enzymes were removed by centrifugation (20,000 × g, 20 min, 4°C). To evaluate the relationship between the amount of Met-Gly and the concentration of the wild-type TabS or the mutants, the reactions were performed for 60 min with various concentration of each enzyme (0.05-0.5 mg/mL) under standard conditions. To determine the kinetic parameters, the reactions were performed with various concentration 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.

Table 5.1. Oligonucleotides used to generate TabS mutants.

Mutation	Oligonucleotide (5' to 3')[a]
H294V	gga ttg GTT cgt ccg gcg gcc aac tat gcg gtc
	cgg acg AAC caa tcc gcc gga caa acg cga ggc
H294L	gga ttg CTT cgt ccg gcg gcc aac tat gcg gtc
	cgg acg AAG caa tcc gcc gga caa acg cga ggc
H294I	gga ttg ATT cgt ccg gcg gcc aac tat gcg gtc
	cgg acg AAT caa tcc gcc gga caa acg cga ggc
H294F	gga ttg TTT cgt ccg gcg gcc aac tat gcg gtc
	cgg acg AAA caa tcc gcc gga caa acg cga ggc
H294W	gga ttg TGG cgt ccg gcg gcc aac tat gcg gtc
	cgg acg CCA caa tcc gcc gga caa acg cga ggc
H294M	gga ttg ATG cgt ccg gcg gcc aac tat gcg gtc
	cgg acg CAT caa tcc gcc gga caa acg cga ggc
H294P	gga ttg CCT cgt ccg gcg gcc aac tat gcg gtc
	cgg acg AGG caa tcc gcc gga caa acg cga ggc
H294G	gga ttg GGT cgt ccg gcg gcc aac tat gcg gtc
	cgg acg ACC caa tcc gcc gga caa acg cga ggc
H294S	gga ttg AGT cgt ccg gcg gcc aac tat gcg gtc
	cgg acg ACT caa tcc gcc gga caa acg cga ggc
H294T	gga ttg ACT cgt ccg gcg gcc aac tat gcg gtc
	cgg acg AGT caa tcc gcc gga caa acg cga ggc
H294Q	gga ttg CAG cgt ccg gcg gcc aac tat gcg gtc
	cgg acg CTG caa tcc gcc gga caa acg cga ggc
H294N	gga ttg AAT cgt ccg gcg gcc aac tat gcg gtc
	cgg acg ATT caa tcc gcc gga caa acg cga ggc
H294Y	gga ttg TAT cgt ccg gcg gcc aac tat gcg gtc
	cgg acg ATA caa tcc gcc gga caa acg cga ggc

[a] Mutated nucleotides are capitalized.

5.2.5. 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 HPLC (L-2000 series; Hitachi High Technologies, Tokyo, Japan). The details of the analytical procedure were described previously (11). Data obtained by HPLC analyses were averages from three independent experiments, and error bars indicated standard deviation of the means.

5.3. Results

5.3.1. Evaluation of the mutants

The author chose to alter Ser85 and His294 residues that might affect enzyme activity of TabS. These residues were replaced with 20 proteogenic amino acids, and dipeptide-synthesizing reactions were conducted using these mutants. The H294T and H294C mutants were not reacted because of their insolubilization. The amounts of phosphate released in the reaction mixtures were measured to select the candidates for the mutants that were able to synthesize Pro-Gly much more than the wild-type TabS (Fig. 5.3, Xaa showed any amino acid).

When Pro and Gly were used as substrates, the reaction mixtures of the S85T, S85C, S85M, S85V, S85L, and S85K mutants contained phosphate equal to or more than that of the wild-type TabS (Fig. 5.3 (A)). Under the same condition, 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. 5.3 (B)). These results indicated that 10 mutants described above might have synthesized Pro-Gly considerably more than the wild-type TabS. Therefore, the amounts of Pro-Gly in the reaction mixtures of

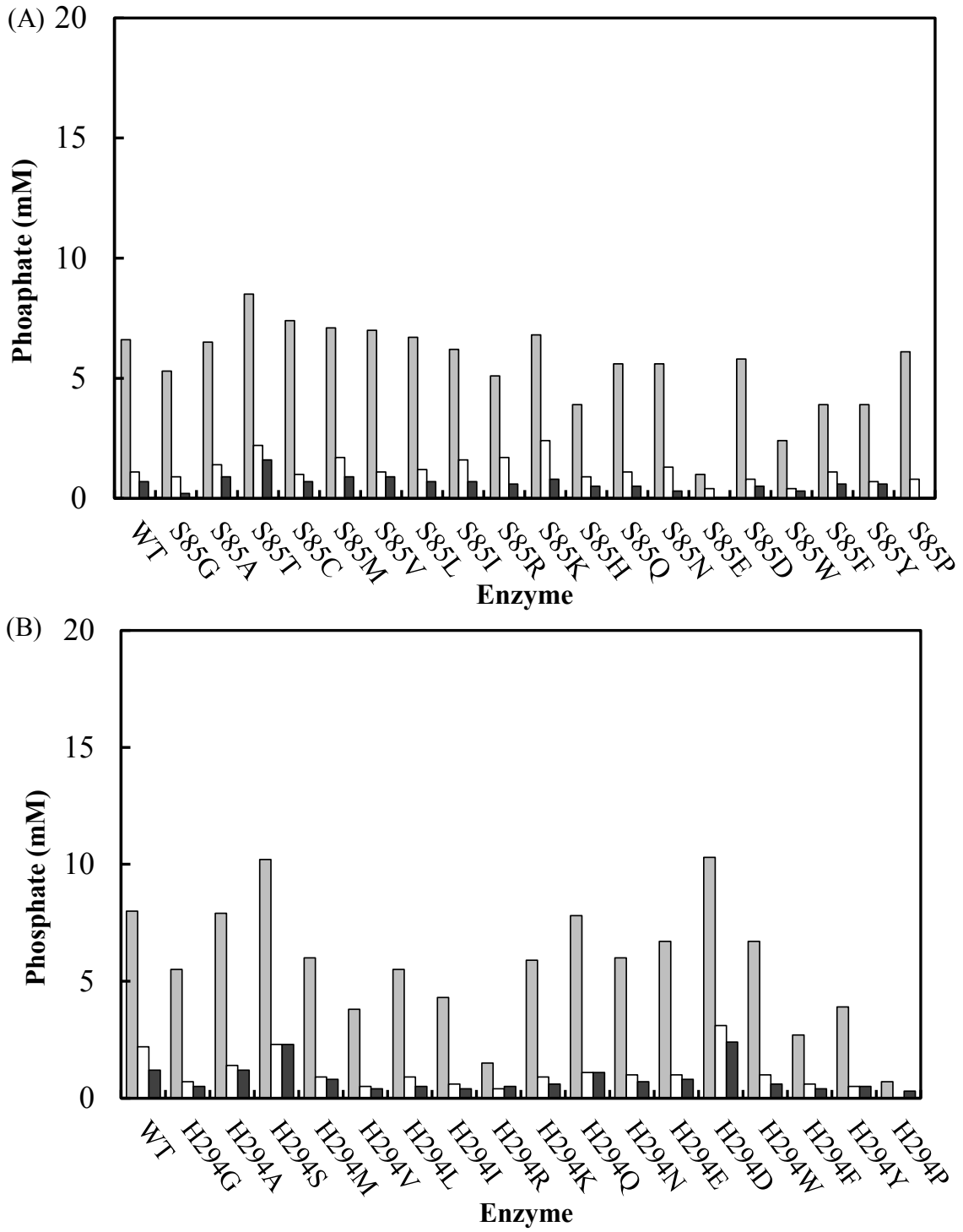

Fig. 5.3. Measurement of the amounts of phosphate produced in reaction mixtures of the wild-type TabS, the S85Xaa (A), and the H294Xaa (B) mutants. Reaction mixture contained 20 mM Met and 20 mM Gly (light gray bar), 40 mM Pro (white bar), 40 mM Gly (dark gray bar), 20 mM ATP, 20 mM $MgSO_4 \cdot 7H_2O$, and 0.5 mg/mL purified TabS or BL00235 in 50 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0). The reaction was performed at 30°C for 20 h.

the mutants were measured by HPLC (Fig. 5.4). 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

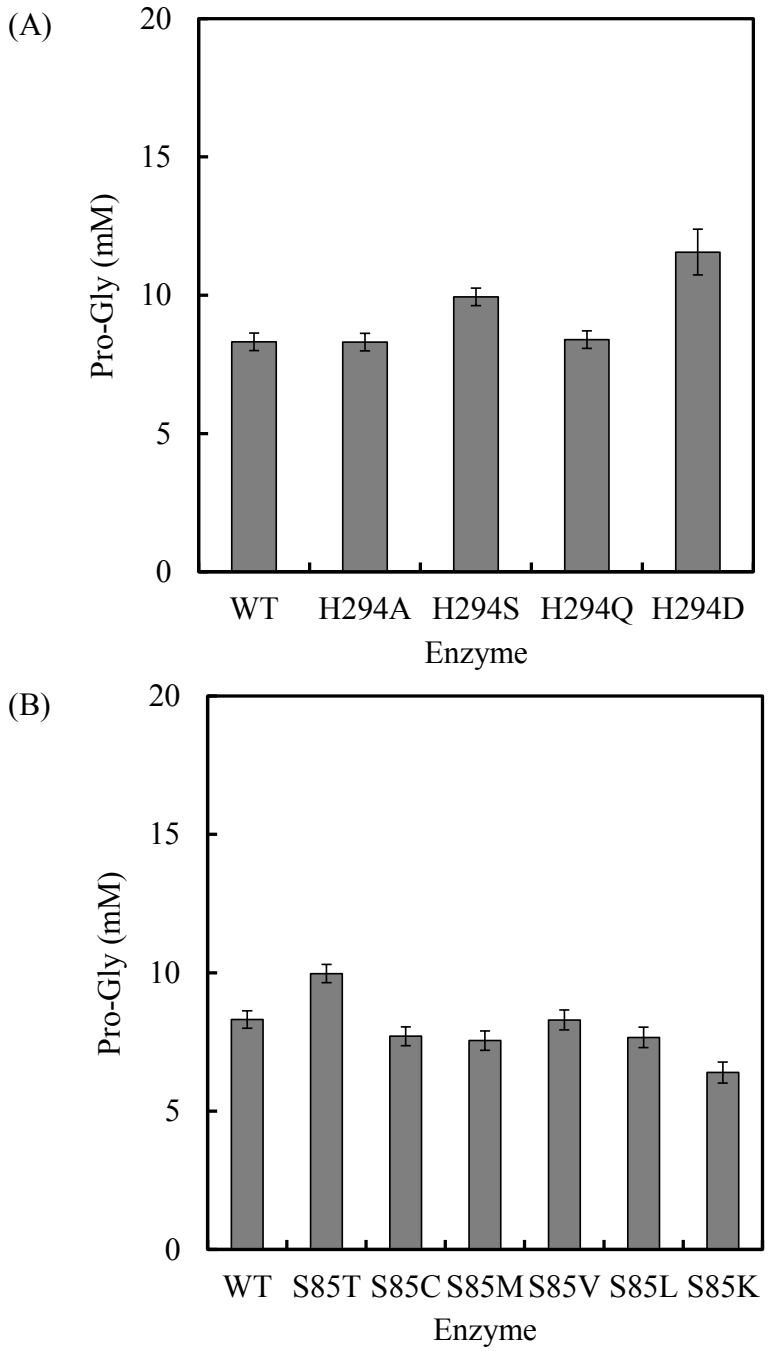

Fig. 5.4. Synthesis of Pro-Gly by the wild-type TabS and the S85Xaa mutants (A) or the H294Xaa mutants (B).

Reaction mixture contained 20 mM Pro and 20 mM Gly, 20 mM ATP, 20 mM $MgSO_4 \cdot 7H_2O$, and 0.5 mg/mL purified enzyme in 50 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0). The reaction was performed at 30°C for 20 h.

the wild-type TabS, respectively. Therefore, the S85T/H294D double mutant was constructed.

5.3.2. Pro-Gly synthesis by the S85T/H294D double mutant

The S85T/H294D double mutant was constructed to increase the amount of Pro-Gly. The dipeptide-synthesizing reaction was performed using 20 mM Pro and 20 mM Gly as substrates (Fig. 5.5). Gly-Pro, Gly-Gly, and Pro-Pro were synthesized in limited amounts by the wild-type TabS and mutants. The S85T/H294D double mutant synthesized Pro-Gly 1.6 times more than the wild-type TabS, and considerably more than the S85T and the H294D mutants.

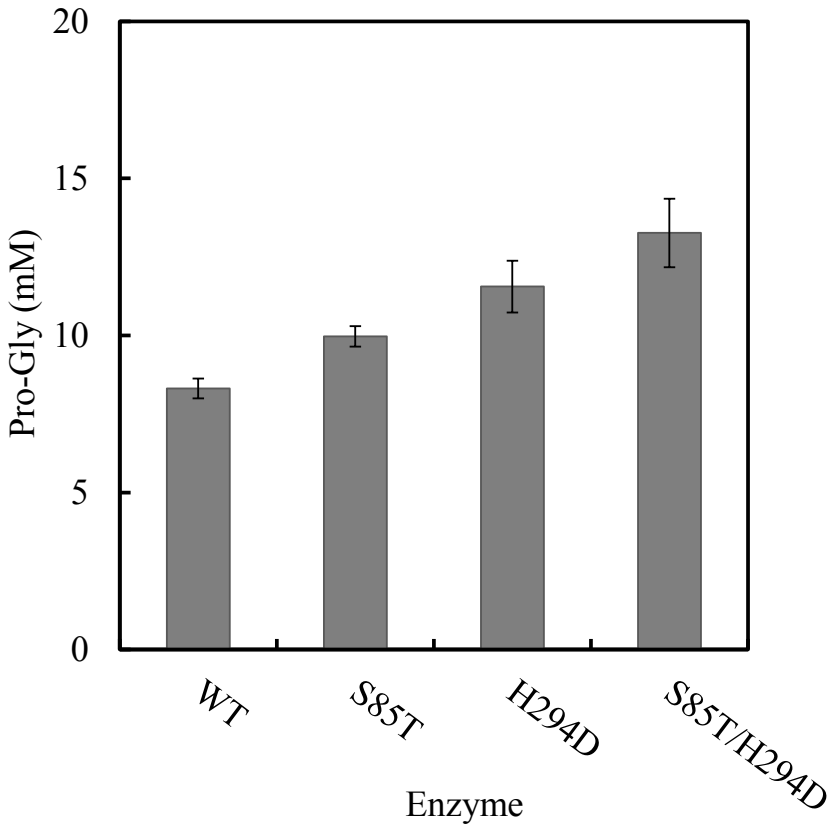

Fig. 5.5. Synthesis of Pro-Gly by the wild-type TabS and the mutants.
Reaction mixture contained 20 mM Pro and 20 mM Gly, 20 mM ATP, 20 mM $MgSO_4 \cdot 7H_2O$, and 0.5 mg/mL purified enzyme in 50 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0). The reaction was performed at 30°C for 20 h.

5.3.3. Characterization of the S85T/H294D double mutant

First, the relationship between the enzyme concentration and the amount of produced Pro-Gly was evaluated (Fig. 5.6). This reaction was conducted for 60 min. the S85T/H294D double mutant exhibited strong activity with each enzyme concentration. Next, the kinetic parameters of Pro-Gly synthesis were determined. According to Hanes-Woolf linearization (Fig. 5.7), the K_ms values and the V_{max} values for Pro-Gly synthesis of the wild type TabS and the S85T/H294D double mutant were shown in Table 5.2. The apparent K_ms values of the S85T/H294D double mutant decreased for Pro and Gly, and increased for ATP more than those of the wild-type TabS, respectively. On the other hand, V_{max} value of the S85T/H294D double mutant was higher than that of the wild-type TabS.

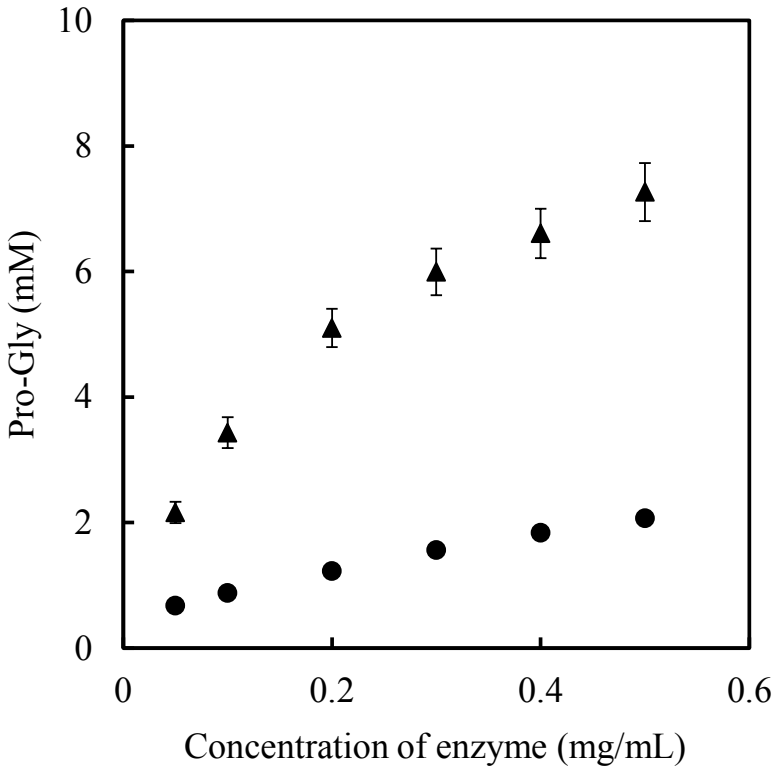

Fig. 5.6. Synthesis of Pro-Gly using various concentration of the wild-type TabS (circle) or the S85T/H294D double mutant (triangle).
Reaction mixture contained 20 mM Pro and 20 mM Gly as substrates. The reaction was performed at 30°C for 60 min.

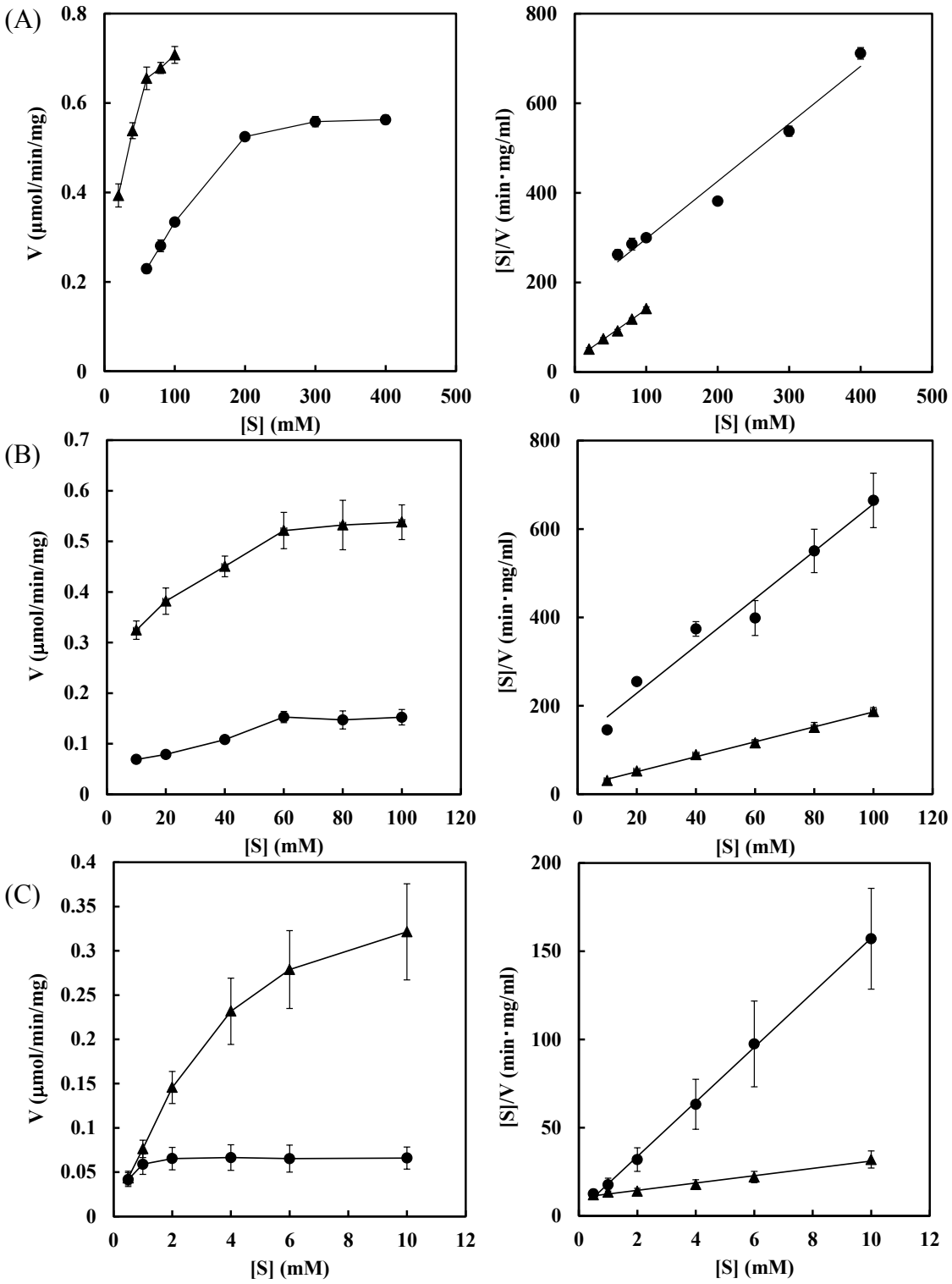

Fig. 5.7. Kinetic analysis of Pro-Gly-synthesizing reaction by the wild-type TabS (circle) and the S85T/H294D double mutant (triangle).

Plots of reaction velocity (V) vs. substrate concentration ([S]) curves (left shapes), and [S]/V vs. [S] curves (Hanes-Woolf plot; right). The reactions were performed by varying the concentration of Pro (A), Gly (B), or ATP (C) at 30°C for 60 min.

Table 5.2. Kinetic parameters of Pro-Gly-synthesizing reaction by the wild-type TabS and the S85T/H294D double mutant.

	*K*m (mM)			*V*max
	Pro	Gly	ATP	(nmol・min^{-1}・mg protein^{-1})
WT	133±15.3	23.3±4.19	0.166±0.109	781±31.3
S85T/H294T	25.1±3.36	10.2±1.07	5.24±1.27	894±24.7

The reaction was performed by varying the concentration of Pro, Gly, and ATP.

5.4. Discussion

In this chapter, the author focused on Ser85 and His294 residues, which positioned around C- and N-terminal substrate and might affect the enzyme activity of TabS, on the basis of findings of BL00235 (2) (Chapter 4) and the predicted structure of TabS (Fig. 5.2). The P85F mutant of BL00235 synthesizes Met-Gly selectively (2), and the Ser85 residue of TabS corresponded to the Pro85 residue of BL00235. When the Ser85 residue was replaced with 20 proteogenic amino acids, the S85T mutant predominantly synthesized Pro-Gly (Fig. 5.4). Ser and Thr differed only in the carbon numbers and have similar properties. In contrast, phosphate analysis showed that the S85H, S85E, S85W, S85F, and S85Y mutants had low Pro-Gly-synthesizing activity (Fig. 5.3 (A)). The author considered that the amino acid residues of bulky side chains at position 85 were not suitable for Pro-Gly synthesizing. These results demonstrated that the amino acid residue at position 85 of TabS is a significant residue for enzyme activity similarly to that of BL00235. When His294 residue was replaced with 20 proteogenic amino acids, the H294D mutant synthesized Pro-Gly the most (Fig. 5.4). According to the phosphate analysis, the H294R and H294P mutants showed limited Pro-Gly-synthesizing activity. The author has not yet determined common properties of

the H294Xaa mutants regarding Pro-Gly synthesis. This could be possible if the structure of TabS was determined. However, these results denoted that the His294 residue of TabS affects the enzyme activity. When the amount of Pro-Gly was measured by HPLC, the S85T/H294D double mutant synthesized Pro-Gly 1.6 times more than the wild-type TabS and superior to the single mutants for 20 h (Fig. 5.5). In contrast, the amount of Pro-Gly synthesized by the S85T/H294D double mutant was 3-4 times as much as that by the wild-type TabS during Pro-Gly synthesis for 60 min (Fig. 5.6). The author considered that the considerable difference of synthesized Pro-Gly at the initial stage of reaction affects the reaction to the end. The apparent K_ms values of the S85T/H294D double mutant for Pro and Gly decreased and that for ATP increased compared with that of the wild-type TabS, respectively (Fig. 5.7). These result showed that the affinity for Pro and Gly for the S85T/H294D mutant is higher and that for ATP is lower than that of the wild-type TabS. While the apparent K_m value of the S85T/H294D double mutant for ATP increased, the amount of Pro-Gly synthesized by the S85T/H294D mutant was considerably more than that of the wild-type TabS. It is possible that the affinity for ATP affects dipeptide synthesis less than that for amino acid substrates. The first step of the mechanism for the dipeptide synthesis catalyzed by Lal is that the N-terminal amino acid binds to the ATP-bound state of the enzyme (3). Therefore, the author considered that the affinity for the N-terminal substrate was the key property to dipeptide synthesis of Lals. Furthermore, increase in V_{max} value of the S85T/H294D double mutant reflected the total Pro-Gly production. The kinetics parameters of Met-Gly synthesis were previously measured using the wild-type BL00235 and the P85F mutant that synthesized Met-Gly selectively, and showed the opposite results in this chapter (2) (Chapter 4). V_{max} value of the P85F mutant was higher than that of the wild-type BL00235, and it is the same

as result from this study. On the other hand, K_ms values were opposite results from this study. For BL00235, the apparent K_m value of the P85F mutant for Met, Gly and ATP were higher than that of the wild-type BL00235, and the amount of Met-Gly synthesized by the P85F mutant was lower than that by the wild-type BL00235. These results indicated that mutagenesis leads to different effects, though the amino acid position 85 affect the enzyme activity in common between BL00235 and TabS.

In this chapter, the author succeeded in increasing the amount of Pro-Gly by constructing the double mutant S85T/H294D based on the findings of BL00235. Results in this chapter and chapter 4 extend the possibilities of Lals, and many useful dipeptides might be able to be synthesized efficiently by applying the concepts and strategies in this chapter to various Lals.

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Chapter 6

Synthesis of L-Prolylglycine Coupled with ATP Regeneration System

6.1. Introduction

Efficient dipeptides synthesis was achieved using L-amino acid ligases (Lals) by site-directed mutagenesis in chapters 4 and 5, and the concepts and strategies shown in the chapters were expected to make Lals easier to use than ever before. However, there are some problems to apply Lals to industrial production process, and one of them is ATP utilization. ATP utilization takes many costs and generates by product such as ADP and AMP (1). In order to solve this problem, ATP regeneration systems were developed using phosphate donors and enzymes such as creatine phosphate and creatine kinase (2), acetyl phosphate and acetyl kinase (3), and polyphosphate and polyphosphate kinase (1, 4, 5).

Polyphosphate kinase (PPK, EC 2.7.4.1) is an enzyme that catalyzes chain-elongating reaction and synthesizes long-chain polyphosphate ($PolyP_n$) from $PolyP_{n-1}$ by transferring the terminal phosphate of ATP (4). PPK is able to synthesize ATP from ADP and $PolyP_n$ because this reaction is reversibly. ATP costs $2000/lb, but $PolyP_n$ provide equivalent amount of ATP for $9/lb; hence ATP regeneration system using $PolyP_n$ and PPK is useful from cost perspective (1). Mainly, two kinds of PPK are known, PPK1 and PPK2. For instance, PPK1 and PPK2 from *Pseudomonas*

aeruginosa have different characteristics. In order to synthesize $PolyP_n$ (forward reaction), PPK1 utilizes ATP only, but PPK2 is able to use ATP and GTP (6). On the other hand, GTP synthesizing reaction (reverse reaction) from GDP by PPK is superior to forward reaction (6). In addition, the new subfamily of PPK2 (Class III PPK2) has been reported recently (7). Class III PPK2 is able to synthesize ATP from AMP and ADP with single reaction, whereas known PPK2 catalyzes ADP or AMP respectively. Suzuki et al. found out that Class III PPK2, Deipr_1912 from *Deinococcus proteolyticus* MRP had activity of ATP regeneration from AMP, and succeeded in synthesizing aminoacyl proline coupled with ATP regeneration system (8).

Though the coupling reaction with Lal and PPK has not been reported so far, Sato et al. constructed ATP regeneration system using D-alanine-D-alanine ligase (TmDdl), and thermostable PPK from *Thermosynechococcus elongates* BP-1 (TePpk) (5). Decreasing the amount of initial ATP led to reduce the amount of D-alanyl-D-alanine synthesized by purified TmDdl only. By contrast, the ability of synthesizing D-alanyl-D-alanine was maintained in coupling with ATP regenerating system using purified TmDdl and TePpk when the amount of initial ATP decreased from 5 mM to 0.005 mM (5). Furthermore, D-alanyl-D-alanine was able to be synthesized without ATP addition in coupling with ATP regenerating system using resting cells (5).

In this chapter, L-prolylglycine (Pro-Gly) synthesis coupled with ATP regeneration system was conducted using the S85T/H294D double mutant of TabS, which synthesized more Pro-Gly than the wild-type TabS (chapter 5), Deipr_1912, and $PolyP_{25}$.

6.2. Materials and methods

6.2.1. Materials

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

6.2.2. Enzyme preparation

Preparations of the S85T/H294D double mutant were described in chapter 2 (see TabS).

The genes encoding Deipr_1912 were previously cloned into the pET21a(+) vectors. Recombinant *Escherichia coli* BL21 (DE3) cells were precultivated in 3 mL LB medium (1% bacto tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 50 μg/mL ampicillin at 37°C for 5 h with shaking at 160 rpm. For the main culture, 150 mL LB medium containing 50 μg/mL ampicillin and 0.1 mM isopropyl-β-D-thiogalactopyranoside was inoculated with 1.5 mL preculture broth, and cultivated with shaking at 25°C for 19 h with shaking on a gyratory shaker (120 rpm). The cells were collected with centrifugation (3,000 × g, 10 min, 4°C) and washed twice with 100 mM Tris-HCl buffer (pH 8.0). After washing, the cells were suspended in 100 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0) and then lysed by sonication at 4°C. The lysate was centrifuged (20,000 × g, 30 min, 4°C), and the supernatant was purified and fractionated with a His Gravitrap^{TM} affinity column (GE Healthcare, Buckinghamshire, UK). Fractions containing protein were desalted with a PD-10 column (GE Healthcare, Buckinghamshire, UK) and Deipr_1912 was eluted with 100 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0).

6.2.3. Pro-Gly synthesis using purified enzyme

The reaction mixtures without ATP regeneration system (300 μL) contained 20 mM Pro and 20 mM Gly as substrates, 0.2 to 20 mM ATP, 20 mM $MgSO_4 \cdot 7H_2O$, and 0.5 mg/mL of the S85T/H294D double mutant in 50 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.0). The reaction mixture coupled with ATP regeneration system (300 μL) contained 20 mM Pro and 20 mM Gly as substrates, 1 mM $PolyP_{25}$, 0.2 to 20 mM ATP, 20 mM $MgSO_4 \cdot 7H_2O$, 0.5 mg/mL of the S85T/H294D double mutant, and 0.1 mg/mL or 0.2 mg/mL of Deipr_1912 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. Enzymes were removed by centrifugation (20,000 × g, 20 min, 4°C).

6.2.4. Analysis

The amount of Pro-Gly was analyzed by HPLC (L-2000 series; Hitachi High Technologies, Tokyo, Japan). The details of the analytical procedure were described previously (9). Data obtained by HPLC analyses were averages from three independent experiments, and error bars indicated standard deviation of the means.

6.3. Results

The Pro-Gly synthesis reaction coupled with ATP regeneration system was conducted (Fig. 6.1). The amount of Pro-Gly decreased remarkably depending on the amount of initial ATP in the reaction mixture without ATP regenerating system. In contrast, there was not much difference of the amount of Pro-Gly coupled with ATP regeneration system regardless of 2 mM and 20 mM initial ATP. Furthermore, when the amount of initial ATP was 0.2 mM, the S85T/H294D double mutant and Deipr_1912 with $PolyP_{25}$ synthesized about 5 mM Pro-Gly. Though we used 0.1 mM

and 0.2 mM Deipr_1912, there was not much different in the amount of Pro-Gly synthesized in the reaction mixture coupled with ATP regeneration system.

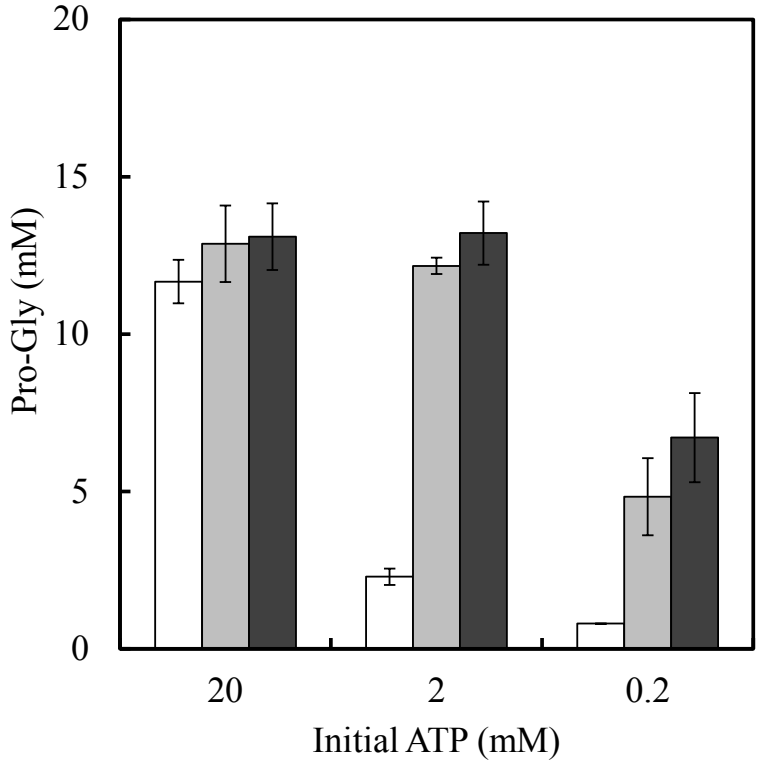

Fig. 6.1. Synthesis of Pro-Gly coupled with ATP regeneration system.
Reaction mixture contained 0.5 mg/mL the S85T/H294D double mutant (white bars), the S85T/H294D double mutant and 0.1 mg/mL Deipr_1912 with 1 mM $PolyP_{25}$ (light gray bars), and 0.2 mg/mL Deipr_1912 with 1 mM $PolyP_{25}$ (dark gray bars). The reaction was performed at 30°C for 20 h.

6.4. Discussion

To reduce ATP utilization and construct reaction system using ATP regeneration system, PPK and $PolyP_n$ were used with Lal in this chapter. The amount of Pro-Gly differed notably between in the reaction mixtures coupled with ATP regeneration system and without the system. There was no difference of the amount of Pro-Gly coupled with ATP regeneration system regardless of 2 mM and 20 mM initial ATP. The 2mM initial ATP was below the K_m value of the S85T/H294D double mutant (chapter

5). D-Alanyl-D-alanine was also synthesized with ATP regenerating system at the same condition (below the K_m value of TmDdl for ATP) (5). These results indicated that ATP was regenerated from ADP using Deipr_1912 and $PolyP_{25}$. Lals do not need use Class III PPK2 because the reaction accompanies the hydrolysis ATP to ADP, not AMP (10). The combination of PPK and $PolyP_n$ are important factors that determine the ability to regenerate ATP (5). Therefore, the amount of initial ATP to operate ATP regeneration system might be reduced less than 2 mM by changing the kinds of PPKs and the length of $PolyP_n$.

In this chapter, the author revealed that dipeptide was synthesized by Lal coupled with ATP regenerating system, and the amount of initial ATP was reduced to 10% at least. The author presumes that further studying on the basis of this finding lead to construct more efficient dipeptides production system from cost perspective.

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Chapter 7

Summary and Conclusion

7.1. Summary

Some dipeptides have unique physiological properties and physiological functions, and the author focused on the taste-improving effect in this thesis. Dipeptide library was constructed using L-amino acid ligase (Lal), and salt taste enhancing dipeptides were screened with new strategy (chapters 2 and 3). Furthermore, these dipeptides were synthesized efficiently by site-directed mutagenesis of Lals (chapter 4-6). The summary was shown as below.

In chapter 1, the author reviewed typical functions of dipeptides, screening methods for functional dipeptides, and evaluation methods for tasty or taste-improving agents. In addition, the author described about Lals containing characteristics, structures, and recent studies. The objective of this thesis was described at the end: (i) screening of salt taste enhancing dipeptides using Lal and (ii) alteration of the substrate specificity of Lal and efficient synthesis of the dipeptides.

In chapter 2, dipeptide library using TabS from *Pseudomonas syringae* (1) was constructed, and two-step screening system was proposed with evaluating the reaction mixtures directly. At that time, the amino acids that were easily released by the hydrolysis of proteins or peptides were selected, and 111 kinds of reaction mixtures containing these amino acids as substrates were synthesized. L-Methionylglycine

(Met-Gly) and L-prolylglycine (Pro-Gly) were found out with sensory assessment as the candidates of new salt taste enhancing dipeptides in the dipeptide library.

In chapter 3, the ability of Met-Gly and Pro-Gly as salt taste enhancers was assessed by sensory assessment and taste sensor analysis using authentic samples, and both evaluations showed that ability. Taste sensor analysis demonstrated that NaCl solution containing Met-Gly had different taste from NaCl solution itself with principal component analysis, and this result corresponded to the sensory assessment. Furthermore, the evaluation that Met-Gly did not have salt taste itself was supported by sensory assessment and taste sensor analysis. The new screening method using Lal is considered to be useful one through chapters 2 and 3. The results in both chapters were summarized in the research paper (2).

In chapter 4, the substrate specificity of BL00235 was altered to synthesize Met-Gly selectivity. BL00235 from *Bacillus licheniformis* (3) is able to synthesize Met-Gly as a major product and L-methionyl-L-methionine (Met-Met) as a by-product when L-methionine (Met) and glycine (Gly) were used as substrates. Pro85 residue of BL00235 was focused on the basis of its structure (PDB ID: 3VOT) (4). The author deduced that Met might not be recognized as a C-terminal substrate if the space around C-terminal substrate is occupied by other amino acid. Hence, Pro85 was replaced with L-phenylalanine (Phe), L-tyrosine (Tyr), and L-tryptophan (Trp) having bulky aromatic side chains by site-directed mutagenesis. All mutants lost the capacity to synthesize Met-Met during the synthesis of Met-Gly. Furthermore these mutants did not synthesize Met-Met, even when Met was used as a substrate. The study in this chapter is the first report of synthesizing a useful dipeptide selectively using Lals by site-directed mutagenesis. The results in this chapter were summarized in the research paper (5).

In chapter 5, the enzyme activity of TabS was altered to increase the amount of Pro-Gly. TabS synthesize Pro-Gly selectivity from its substrate specificity, but the yield is low. The author deduced that the amino acid residue at position 85 had a key role in the enzyme activity from chapter 4 and applied these findings to TabS. Ser85 residue of TabS that corresponds to Pro85 residue of BL00235 was selected as the amino acid residue affecting the enzyme activity. His294 residue, which positioned around N-terminal substrate and might be affect the enzyme activity, was also selected on the basis of TabS modeling structure and information of YwfE (PDB ID: 3VMM), from *Bacillus subtilis* (6, 7). 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 the wild-type TabS. Furthermore, the S85T/H294D double mutant synthesized Pro-Gly considerably more than each single mutant. These results showed that the amino acid residue at position 85 affects the enzyme activity in common between TabS and BL00235. The results in this chapter were summarized in the research paper (8).

In chapter 6, Pro-Gly was synthesized using the S85T/H294D double mutant coupled with ATP regeneration system to reduce the amount of costly ATP. Polyphosphate (PolyP_n) is inexpensive, hence, Polyphosphate kinase (PPK) and PolyP_n were used to generate ATP from ADP. The amount of Pro-Gly was decreased remarkably dependent on the amount of initial ATP without ATP regeneration system. In contrast, the ability of synthesizing Pro-Gly was maintained coupled with ATP regeneration system even if initial ATP was reduced to 10%. These results showed that more efficient dipeptides production system was constructed from cost perspective.

In chapter 7, the author describes the summary of this thesis and the perspectives in future.

7.2. Conclusion

In this thesis, industrial and academic knowledge of Lals was obtained. In the first half of this thesis, dipeptide library was constructed using Lal, and new salt taste enhancing dipeptides, Met-Gly and Pro-Gly, were found out in the dipeptide library. These findings are industrially useful ones because development of salt taste enhancing agents is expected with the social back ground of health-conscious. The author's group is now developing proteolytic or microbial digests of natural proteins containing Met-Gly or Pro-Gly sequence to practical use. Met-Gly and Pro-Gly were evaluated only in the NaCl solution in this thesis, hence, changing model materials like instant processed food, noodle-soups and seasonings and using the dipeptides with other substrates may lead to enhance the salt taste intensities strongly. Furthermore, the author considers that this new screening method using Lal is applicable for other taste evaluation of dipeptides such as bitter-masking effect.

In the latter half of this thesis, the effective production of dipeptides was constructed using Lals. No study has been demonstrated that changing the substrate preferences of Lals by a single mutation on the basis of the structure leads to synthesize useful dipeptides so far. The author showed the key amino acid residues in the enzyme activity and succeeded in synthesizing Met-Gly selectivity and increasing the amount of Pro-Gly by site-directed mutagenesis of Lals. These achievements are significant findings in the academic field. They are also useful in the industrial field because the problems of by-products-synthesizing and low yield are resolved. In addition, dipeptide-synthesizing system coupled with ATP regeneration system was constructed and contributes to cost reduction. The utilization of recombinant microorganisms for producing useful stuff is limited, and it is not easy that dipeptides, which are synthesized by recombinant Lals produced by *Escherichia coli*., are used for

foodstuffs in Japan now. However, the Ministry of Economy, Trade and Industry now beginning to allow fragrance production to use recombinant microorganisms (9), and there is a possibility of applying this concept to food stuffs like food additives in near future. The author believes the results of this thesis serve as a window to synthesize dipeptides freely.

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研究概要

ジペプチドはアミノ酸 2 個からなる単純な構造であるが、血圧降下作用を有する Ala-Phe や Ile-Trp、抗不安・ストレス緩和作用を有する Tyr-Leu などそれを構成するアミノ酸単体には認められない生理活性や機能性を有するものが知られている。スクロースの 200 倍の甘さを有するアスパルテーム（Asp-Phe-OMe）は良く知られている呈味性ジペプチドであり、カフェインなどの苦味に対しマスキング作用を有する Glu-Glu や、コク味を増強させる γ-Glu-Leu など、ジペプチド自体には呈味はないが呈味改善作用を有するジペプチドも存在する。そこで申請者は呈味の中でも「塩味」に着目した。食塩は人間にとって必要不可欠な成分であるが、一方、その過剰摂取は高血圧症や心臓疾患などの疾病を引き起こすことが知られている。我が国では 1 日当たりの食塩摂取量の目標値は 8.0 g と設定されているが、「減塩」への意識から年々減少傾向ではあるものの、平成 25 年の男女平均値は 10.2 g/day と目標値にはほど遠い現状にある。こうした社会的背景を踏まえ、塩味を呈する物質の開発研究が盛んに行われている。塩味増強効果を有するジペプチドとして Leu-Ser や Glu-Thr などの報告があるが、まだその効果が満足できるジペプチドはない。そこで、申請者はジペプチドの機能多様性に期待して、塩味増強効果を有する新たなジペプチドの探索を検討することにした。

これまで機能の知られているジペプチドの多くは天然のタンパク質を酵素や微生物で加水分解したものから調製している。具体的には、目的の機能を有する分解物の画分からその機能を担うジペプチドの配列を決定する方法で見出されている。この方法では、加水分解によって遊離しやすいアミノ酸を含むジペプチドは存在量が少ないため評価対象とならず、それらジペプチドの中には有用機能を有するものがまだ多く存在するのではないかと考えるに至った。つまり分解物を評価するのではなく、ターゲットとするジペプチドを直接合成して評価する新たな方法論を提唱した。そしてジペプチドの合成には、任意のジペプチドが合成可能でかつ合成したジペプチドを精製することなく反応液のまま迅速に評価できる L-アミノ酸リガーゼ（Lal）を用いることを考えた。

Lal は無保護のアミノ酸同士を ATP の加水分解反応と共役して直接連結することを特徴とする酵素であり、これまでに十数種類の Lal が報告されている。Lal により基質特異性は大きく異なるため、本検討では基質特異性の広い *Pseudomonas syringae* 由来の TabS を用いてジペプチドを合成した。また、ターゲットとするジペプチドは、各種タンパク質の加水分解で生じる遊離アミノ酸データよりアミノ酸を選抜し、これらアミノ酸を含むジペプチドを中心に合成し、評価を行った。本論文の第 1 章から第 3 章では、TabS によるジペプチドライブラリーの構築と塩味増強効果を有するジペプチドの探索方法の構築、そして本探索

により見出した候補ジペプチドの評価について論じた。さらに、第 4 章から第 6 章では、塩味増強効果を有するジペプチドの効率的合成法の確立を目的として、Lal の結晶構造情報を利用した部位特異的変異導入と改変 Lal によるジペプチドの選択的合成と生産性向上、ならびに ATP 再生系を導入した低コストプロセスについて論じた。Lal の改変によるジペプチドの選択的合成の成功は本研究が初めての報告であり、Lal の工業的利用と酵素学的知見について記述した。

本論文は 7 章で構成されている。

第 1 章では、ジペプチドの有する機能性と呈味改善素材の探索や評価方法及び Lal について概説した。さらに、本研究の戦略と意義についても述べた。

第 2 章では、Lal の一種である TabS を用いてジペプチドライブラリーを構築し、塩味増強効果を有するジペプチドの候補を選抜した。はじめに、従来の機能性ジペプチドの探索では対象とならなかったと予想されるジペプチドを中心に 111 種類の反応液を調製した。ここで、反応液をそのまま評価試料とするスクリーニング方法を構築し、迅速な評価を可能とした。評価試験により、Met-Gly と Pro-Gly を塩味増強効果を有するジペプチドの候補として選抜した。

第 3 章では、Met-Gly と Pro-Gly の標品を用いて、官能評価と塩味センサーによる塩味増強効果の評価を行った。官能評価では 0.5～0.8% (w/v)の食塩水と各ジペプチドを 0.1% (w/v)添加した 0.6% (w/v)食塩水（試料溶液）をブラインドで塩味の強い順に並び替え、試料溶液の塩味の強さがどの食塩濃度に相当するかを熟練したパネルが評価した。いずれの試料溶液も 0.6～0.65% (w/v)の食塩濃度に相当し、両ジペプチドの塩味増強効果が確認できた。また、客観的評価として味覚センサー（アルファ・モス製）による評価も実施し、食塩水と試料溶液の塩味相対強度から、Met-Gly は 0.7% (w/v)、Pro-Gly は 0.64% (w/v)の食塩濃度に相当し、0.6% (w/v)食塩水よりも高い食塩濃度に相当する結果を得た。両ジペプチドともこれまでに塩味増強効果に関する報告は無く、官能評価と味覚センサーの 2 つの異なる方法により両ペプチドの塩味増強効果が確認されたことから、Met-Gly と Pro-Gly は塩味増強効果を有する新規なジペプチドであると判断した。

第 4 章では、塩味増強効果を有するジペプチドとして見出した Met-Gly の選択的合成を目的に、部位特異的変異導入による Lal の機能改変を行った。Met-Gly の工業的生産には、N 末端アミノ酸基質として Met と Leu のみを認識する *Bacillus licheniformis* 由来の Lal である BL00235 を用いることとしたが、Met と Gly を基質とする反応では Met-Gly が主生成物となるが Met-Met も同時に生成する。そこで、既に明らかになっている BL00235 の結晶構造情報から、85 位の Pro 残基が C 末端アミノ酸基質の親和性に関与していると推測し、Met-Met の合成を抑制するための方法を策定した。すなわち、85 位の Pro 残基を Pro よりも嵩高いアミノ酸に置換することで C 末端アミノ酸基質認識周辺のスペースが空間的に狭くなり、その結果、側鎖の大きい Met は認識されず、側鎖の小さい Gly のみが選択的に C 末端基質

として認識されると考えた。実際に部位特異的変異導入によって Pro よりも嵩高い側鎖を持つ芳香族アミノ酸である Phe、Tyr、Trp に置換したところ、その変異酵素 P85F、P85Y、P85W は Met-Met 合成能力が消失し、P85F と P85Y では Pro-Gly 合成活性を維持していた。これらの結果は、Lal の構造情報から推測した特定のアミノ酸残基の一置換変異によって C 末端アミノ酸の基質認識が予想したように変化したことを示すものである。結晶構造情報に基づいて改変した Lal による目的ジペプチドの選択的合成の成功は初めての報告となる。置換により C 末端アミノ酸基質認識周辺のスペースが狭くなっていることはホモロジーモデリングによる構造予測からも視覚的に確認することができた。また、Met-Gly の合成量は野生型 BL00235 よりも P85F の方が少ないが、動力学的解析からも基質との親和性が野生型 BL00235 よりも低いことが示唆され、合成量の違いを支持する結果を得た。

第 5 章では、第 4 章での知見が他の Lal にも適用可能であるかを検証するために、塩味増強ジペプチドとして Met-Gly とともに見出された Pro-Gly をターゲットとして TabS の機能改変を行った。TabS は基質特異性から Pro と Gly を基質としたときに選択的にほぼ Pro-Gly のみを合成するが、その合成量は、基質である 20 mM の Pro と Gly 対し 10 mM 以下と少ない。そこで、部位特異的変異導入による Pro-Gly の合成量向上を検討した。TabS は結晶構造が解かれていないため、第 4 章の BL00235 を含むこれまでの Lal の構造と機能に関する知見を踏まえ、C 末端アミノ酸基質の親和性に関与するアミノ酸残基として BL00235 で親和性に関わる Pro85 に相当する Ser85 と、N 末端基質アミノ酸基質の親和性に関与するアミノ酸残基として His294 に着目し、それぞれに対しサチュレーション変異導入を行った。その結果、Thr に置換した S85T と Asp に置換した H294D で Pro-Gly の合成量が野生型 TabS よりも増加し、BL00235 と同様に 85 位のアミノ酸残基が基質アミノ酸に関与することを確認した。さらにこの 2 つの変異を掛け合わせた二重変異型酵素 S85T/H294D では Pro-Gly の合成量が一変異型よりもさらに増加し、Pro-Gly の合成に適した改変型 TabS の取得に成功した。動力学的解析では二重変異型酵素 S85T/H294D は Pro と Gly に対する親和性が野生型 TabS よりも高まっており、Pro-Gly の合成量の増加を支持する結果を得た。

第 6 章では、Lal の反応に用いる ATP が高価であることから、ATP の安価かつ効率的な供給を目的として、ポリリン酸キナーゼとポリリン酸による ATP 再生系の利用可能性を検討した。ポリリン酸キナーゼとして Class III PPK2 の活性が報告されている *Deinococcus proteolyticus* 由来の Deipr_1912 を利用したところ、ATP の使用量を従来の 1/10 にした場合、再生系を導入していない系では顕著に Pro-Gly の合成量が減少するのに対し、再生系を導入した系では Pro-Gly の合成能力を維持した。この結果から、Lal を利用したジペプチド合成反応において ATP の使用量を大幅に削減できる低コストプロセス構築の可能性を示した。

第 7 章では、本研究を総括した。本研究では、Lal を用いた機能性ジペプチドの探索と結晶構造情報を利用した酵素のデザインと改変による目的ジペプチドの効率的合成法の開発に成功した。また、Lal 研究の今後の展望について述べた。

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梅澤覚, 角谷政尚, 服部宏一, 東條博昭, 駒井強, 斉藤司, 木野はるか, 木野邦器

その他

1. 特許：特願 2014-061601「塩味増強剤」木野はるか, 角谷政尚, 服部宏一, 木野邦器

2. 特許：特願 2015-128323「塩味増強剤」木野はるか, 角谷政尚, 服部宏一, 木野邦器

3. 受賞：日本農芸化学会 2015 年度大会トピック賞

4. 記事：学会見聞記, バイオサイエンスとインダストリー , **73**, 54 (2015)

5. 記事：「塩味増強効果のジペプチド　酵素合成法で迅速探索　長谷川香料　新食材開発に活用」
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6. HP：長谷川香料（株）研究情報　L-アミノ酸リガーゼの機能改変による塩味増強ジペプチドの選択的合成に成功
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