Characterization of Amino Acid Ligases and Development of Production Processes for Peptide Containing D-Amino Acid

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Thesis for the Doctor's Degree in Technology
Accepted by Waseda University

Tokyo, March 2007

Preface

Peptides were discovered as antimicrobial compounds and signal molecules in nature, and are utilized as functional foods and drug materials. Although there are few natural peptides that contain D-amino acids, peptides containing D-amino acids (D-amino acid peptides) are expected to increase the functional variety and applications of peptides. Therefore effective production processes of D-amino acid peptides are desired for further studies concerning to function and application of these peptides.

In this thesis, with the objective to develop effective production processes for D-amino acid peptides, amino acid ligases with broad substrate specificity were explored based on diversity of bacteria, and effective D-amino acid dipeptide synthesis utilizing amino acid ligase coupled with amino acid racemase or ATP regeneration systems was performed. I hope that the studies in this thesis will provide useful information for not only the D-amino acid peptide production, but also the studies in the fields of microbiology, biotechnology, and applied chemistry.

Masaru Sato

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Chapter 1

REVIEW:
Enzymatic Production of D-Amino Acid Peptides

1.1. Introduction

Peptides are discovered as antibacterial compounds and signal molecules in nature. Because of these diversity of natural peptides, the studies concerning to development for various peptide usages as pharmaceuticals and functional materials are preceded. In the conventional studies on peptide usages, chemical modifications for natural peptides with certain functions have been attempted to obtain more suitable peptide derivatives. On the other hands, the development of the methods for peptide synthesis and the high-throughput analysis of peptide functions enabled the comprehensive and rapid analysis of diverse peptides. Subsequently, the functions of unnatural peptides, that contain non-standard amino acid such as D-amino acid and unnatural amino acid, were focused.

In the synthesis of D-amino acid containing peptides, reverse reaction of D-enantiomer specific peptidases and esterases were considered as possible methods to synthesize those peptides. However, these enzymes utilize D-amino acid esters and amides as substrates, and the reverse reaction must be carried out under the restrict reaction conditions. Therefore, other enzymes that synthesize D-amino acid peptides from free amino acids under the easy reaction conditions are needed.

In this chapter, overview of enzymes concerning to D-amino acid containing

peptides was described and the problems in previous methods to synthesize those peptides were clarified. Besides, the studies in D-alanyl-D-alanine ligase, which was focused as another possible enzyme for D-amino acid containing peptide synthesis in this thesis, were introduced. Based on the review in this chapter, the objective of this thesis was clarified.

1.2. Enzymes acting on peptide containing D-amino acid

1.2.1. D-Aminopeptidase

The first example of enzymatic synthesis of D-amino acid containing peptide was demonstrated using D-aminopeptidase from *Ochrobactrum anthropi* by Kato *et al.* (1). This enzyme has been obtained by Asano *et al.*, and clarified that it showed strict D-stereospecificity toward substrates including low molecular weight D-amino acid amides, D-alanine *N*-alkylamides, and peptides with a D-alanine at the N-terminus (2). The detailed substrate specificity of the peptidase was summarized in TABLE 1.1 and 1.2.

Because the reverse reaction of the peptidase progressed under the condition with organic solvents, the effect of tertiary amines *i.e.* triethylamine, di-isopropylethylamine, tripropylamine, tributylamine, and *N,N*-dimethylaminopyridine on the reverse reaction was examined. Among these tertiary amines, it was revealed that triethylamine was the most effective solvent, and it was demonstrated that D-alanyl-D-alanine and D-alanine trimer were synthesized from D-alanine methyl ester in the yield of 58% and 6%, respecrively.

TABLE 1.1 Substrate specificity of D-aminopeptidase from *O. anthropi* SCRC C1-38 (2)

Substrate	Relative activity (%)	Km (mM)	Vmax (units/mg)
D-Alanine amide	100	0.65	600
Glycine amide	44	22.3	365
D-α-Aminobutyric acid amide	30	18.3	576
D-Serine amide	29	27.0	22.0
D-Threonine amide	9	100	60.3
D-Methionine amide	2		
D-Norvaline amide	1.8		
D-Norleucine amide	0.8		
D-Phenylglycine amide	0.7		
D-Alanylglycine	95	0.98	1000
D-Alanylglycylglycine	45	0.37	799
D-Alanyl-D-alanine	21	10.2	326
D-Alanyl-D-alanyl-D-alanine	92	0.57	866
D-Alanyl-D-alanyl-D-alanyl-D-alanine	89	0.32	702
D-Alanyl-L-alanine	46	1.03	312
D-Alanyl-L-alanyl-L-alanine	100	0.65	730
D,L-Alanyl-D,L-serine	27		
D,L-Alanyl-D,L-methionine	20		
D,L-Alanyl-D,L-phenylalanine	9		
D,L-Alanyl-D,L-asparagine	7		
D,L-Alanyl-D,L-leucine	1		
D,L-Alanyl-D,L-valine	229		
Glycine methyl ester	0.5		
D-Alanine methyl ester	75		
D-Alanine-*p* -naphthylamide	32		
D-Alanine benzylamide	72	0.51	768
D-Alanine anilide	73		
D-Alanine-*p* -nitroanilide	96	0.51	696
D-Alanine N-butylamide	66	0.73	670
D-Alanine-3-aminopentane amide	32	2.27	288
D-Alanine N-laurylamide	19		
D-Threonine benzyl ester	3.2		

TABLE 1.2 L-Amino acid derivatives active as substrates (2)

Substrate	Relative activity (%)
L-Serine amide	1.1
L-Threonine amide	0.5
L-Alanylglycine amide	3.0[a]
L-Alanine methyl ester	53
L-Serine methyl ester	26
L-α-Aminobutyric acid methyl	2.0
L-Threonine methyl ester	1.6
L-Alanine-*p* -nitroanilide	42

[a] Determined by the formation of ammonia. No formation of L-alanine was detected.

1.2.2. Alkaline D-peptidase

Asano *et al.* purified a novel extracellular D-stereospecific endopeptidase, alkaline D-peptidase (D-stereospecific peptide hydrolase, EC 3.4.11.), to homogeneity from the culture broth of the soil bacterium *Bacillus cereus* strain DF4-B (3). The enzyme was strictly D-stereospecific toward oligopeptides and showed the substrate specificity shown in TABLE 1.3. Athough carboxypeptidase or DD and D-aminopeptidase activities were undetectable, it was clarified that the enzyme was D-stereospecific dipeptidyl endopeptidase from the mode of the hydrolysis of D-Tyr-(D-Phe)$_2$ and (D-Phe)$_2$-D-Tyr.

On the basis of this peptidase, Komeda *et al.* demonstrated the synthesis of (D-Phe)2 from D-phenylalanine methyl ester or D-phenylalanine amide, and investigated the synthesis of D-phenylalanine oligomer (4). However, the D-phenylalanine oligomer synthesis strictly depended on pH and concentration of substrate. In the optimized reaction conditions, 50 mM of the substrate was incubated for 8 h with ADP

2.0 U/ml and 0.4 U/ml, respectively, in 100 mM triethylamine-HCl (pH 11.5), (D-Phe)$_2$ and (D-Phe)$_3$ were produced in 25.4% and 8.6% yield, respectively. Besides, addition of dimethylsulfoxide to the reaction mixture was needed in the production of (D-Phe)$_4$ (6.7% yield).

TABLE 1.3 Substrate specificity of alkaline D-peptidase (3)

Substrate	Relative activity (%)	Km (mM)	Vmax (units/mg)	Vmax/Km (units/mg/mM)
(D-Phe)$_6$	1.8[a]			
(D-Phe)$_4$	100[a]	0.398	199	500
(D-Phe)$_3$	90[a]	0.127	130	1020
(D-Phe)$_2$	0.2[b]	50.1	13.7	0.27
D-Phe-L-Phe	<0.1[b]			
(D-Phe)$_2$-L-Phe	14.9[a]	0.522	30.6	59
L-Phe-(D-Phe)$_2$	119[c]	0.455	154	346
L-Phe-D-Phe-L-Phe	28.1[c]	1.63	66	41
D-Tyr-(D-Phe)$_2$	83.6[b]			
(D-Phe)$_2$-D-Tyr	83.6[a]			
D-Phe-OMe	15,[a] 1.8[b]			
D-Phe-NH$_2$	0.1,[a] 0.1[b]			
D-Phe-pNA	4.2b			
Boc-(D-Phe)$_4$	1.8,[d] 0.8[e]			
Boc-(D-Phe)$_3$	3.2,[d] 1.1[e]			
Boc-(D-Phe)$_2$	7.0[d]			
Boc-(D-Phe)$_3$-OtBu	1.2,[d] 0.3[e]			
Boc-(D-Phe)$_4$-OMe	0.5,[d] 0.2[e]			
Boc-(D-Phe)$_3$-OMe	0.7,[d] 0.3[e]			
Boc-(D-Phe)$_2$-OMe	1.4[d]			
Ampicillin	8.9[f]	73.1	262	3.58
Penicillin G	9.7[f]	48.9	250	5.11

[a] Formation of (D-Phe)$_2$.

[b] Formation of D-Phe.

[c] Formation of L-Phe-D-Phe.

[d] Formation of Boc-D-Phe.

[e] Formation of Boc-(D-Phe)$_2$.

[f] Consumption of a β-lactam compound was measured.

1.2.3. α-Amino acid esterase

An α-Amino acid esterase was obtained from *Bacillus mycoides* as the enzyme acting on D-amino acid containing peptides by Sugihara *et al.*. (5). The α-amino acid esterase was purified to homogeneity, and showed substrate specificity for methyl esters of D,L-form phenylalanine, tryptophan, tyrosine, aspartate, methionine (TABLE 1.4). Besides, this enzyme activity was inhibited by addition of PMSF, therefore it was suggested that serine residue was contained in the active site of this enzyme. Amino acid esterase containing serine residue in its active site was known to show peptide synthesizing activity from amino acid ester by means of ester exchange reaction. Then, synthesis of D-amino acid peptide utilizing the α-amino acid esterase was examined.

TABLE 1.4 Substrate specificity of α-amino acid esterase (4)

Substrate	Hydrolytic activity (%)	Substrate	Hydrolytic activity (%)
D-Phe-OCH_3	100	Methyl caproate	2.0
L-Phe-OCH_3	99	Methyl caprylate	1.1
Ac-D-Phe-OCH_3	119[a]	Methyl laurate	0.3
Ac-L-Phe-OCH_3	111[a]	Methyl oleate	0.5
D-Trp-OCH_3	96	D-Phe-NH_2	3.6
L-Trp-OCH_3	96	D-Phe-D-Phe-OH[b]	ND
D-Tyr-OCH_3	93	D-Phe-L-Phe-OH[b]	ND
L-Tyr-OCH_3	95	L-Phe-D-Phe-OH[b]	ND
D-Asp(OCH_3)-OCH_3	107	L-Phe-L-Phe-OH[b]	ND
L-Asp(OCH_3)-OCH_3	106	L-Ala-L-Ala-OH	ND
D-Met-OCH_3	102	D-Ala-D-Ala-OH	ND
L-Met-OCH_3	98	D-Phe-L-Ala-OH	ND
Methyl acetate	1.1	L-Phe-L-Ala-OH	ND
Methyl propionate	2.7	L-Ala-Gly-OH	ND
Methyl butyrate	3.6	Casein	ND

The following amides were inert as substrates: amides of D-/L-Ala, D-/L-Arg, D-/L-Asp, D-/L-Asn, D-/L-Gln, D-/L-Glu, D-/L-His, D-/L-Ile, D-/L-Leu, D-/L-Lys, D-/L-Met, L-Phe, D-/L-Pro, D-/L-Ser, D-/L-Thr, D-/L-Trp, D-/L-Tyr, D-/L-Val, and Gly. The relative activity was expressed as a percentage of the activity obtained with D-Phe-OCH_3 as the substrate.
ND: not detected. [a] In the presence of 20% DMF. [b] In the presence of 10% DMF.

As a result, it was confirmed that this enzyme was capable to synthesize D-amino acid peptides from esters of aromatic D-amino acid and D-aspartate from LC/MS analysis (TABLE 1.5). It was also revealed that the chain length of the synthesized peptides was two to six and seven or eleven residues from aromatic D-amino acid and D-aspartate, respectively. In the comparison with other enzymes acting on D-amino acid peptides reported so far, this enzyme was superior to them as for the length of the peptide synthesized.

TABLE 1.5 Retention times, molecular masses and proposed structures of the reaction products derived from D-Phe-OCH_3, D-Trp-OCH_3, D-Tyr-OCH_3, and D-Asp(OCH_3)-OCH_3 (5)

Substrate	Retention time (min)	Molecular Mass[a] (Da)	Structure[b]
D-Phe-OCH_3	5.7	312	(D-Phe)$_2$-OH
	6.7	326	(D-Phe)$_2$-OCH_3
	7.0	460	(D-Phe)$_3$-OH
	8.0	473	(D-Phe)$_3$-OCH_3
	8.9	620	(D-Phe)$_4$-OCH_3
	9.6	768	(D-Phe)$_5$-OCH_3
	10.4	915	(D-Phe)$_6$-OCH_3
D-Trp-OCH_3	7.0	404	(D-Trp)$_2$-OCH_3
	8.1	591	(D-Trp)$_3$-OCH_3
D-Tyr-OCH_3	3.6	344	(D-Tyr)$_2$-OH
	4.4	358	(D-Tyr)$_2$-OCH_3
	4.6	522	(D-Tyr)$_3$-OCH_3
	5.2	683	(D-Tyr)$_4$-OCH_3
D-Asp(OCH_3)-OCH_3	6.1	932	(D-Asp(OCH_3))$_7$-OCH_3
	7.3	1,446	(D-Asp(OCH_3))$_{11}$-OCH_3

[a] Determined by FAB mass spectrometry. [b] Determined by amino acid analysis and FAB mass spectrometry.

Moreover, as shown in TABLE 1.6, this enzyme also recognized L-amino acid amides and synthesized D,L-configuration peptides. A number of enzymes specifically acting on D-amino acid-containing peptides has been isolated, and some of these enzymes were shown to hydrolyze peptides of D,L- as well as D,D-configuration. However, there have been no examples of the synthesis of peptides of D,L-configuration by use of these enzymes. Therefore, this was the first report concerning to synthesis of D,L-configuration peptides.

TABLE 1.6 Retention times on HPLC, masses, and yields of the products derived from Ac-D-Phe-OCH_3 and D-/L-Phe-NH_2, or D-/L-Leu-NH_2 (5)

N-Component	Product[b]	Retention time[c] (min)	Mass[d] (Da)	Yield (%)
D-Phe-NH_2	Ac-D-Phe-D-Phe-NH_2	6.3	353	4.7
L-Phe-NH_2	Ac-D-Phe-L-Phe-NH_2	6.6	353	21.3
D-Leu-NH_2	Ac-D-Phe-D-Leu-NH_2	5.8	319	2.1
L-Leu-NH_2	Ac-D-Phe-L-Leu-NH_2	6.3	319	4.8

[a][Ac-D-Phe-OCH_3] = 150 mM, [N-component] = 300 mM, with 20 units of enzyme at pH 7.0 and 25ºC for 10 min in the presence of 20% DMF.
[b]Deduced from the results of amino acid analysis and FAB mass spectrometry.
[c]Obtained by HPLC. [d]Determined by FAB mass spectrometry.

1.2.4. Aminoacyltransferase

Saccharothrix sp. AS-2 was isolated from soil as a microorganism that produces D-phenylalanine oligomer from D-phenylalanine methyl ester (6). It was revealed that the D-phenylalanine oligomer production was catalyzed by aminoacyltranferase, and the enzyme was purified to homogeneity. Although peptidase and esterase, *i. e.* alkaline D-peptidase and α-amino acid esterase, showed hydrolyzing activity toward amino acid esters, there was no detectable hydrolyzing activity for amino acid esters with the aminoacyltransferase. Thus, it was considered that this characteristic was unique and

useful for D-amino acid peptide production.

Then, Sugihara *et al.* investigated the substrate specificity of the aminoacyltransferase, and revealed that methyl esters of D-phenylalanine, D-tryptophan, D-tyrosine, D-aspartate, and L-glutamate were recognized as substrates (TABLE 1.7) (7). It was interesting finding that L-glutamate methyl ester was recognized by the aminoacyltransferase, since it was supposed to be a D-specific enzyme. However, it was unclear why L-form was recognized only in the case of glutamate.

TABLE 1.7 Aminoacyltransferase activity toward α-amino acid methyl esters (7)

Substrate	Activity (%)
D-Phe-OMe	100
L-Phe-OMe	0
D-Asp(OMe)-OMe	91
L-Asp(OMe)-OMe	0
D-Trp-OMe	14
L-Trp-OMe	0
D-Tyr-OMe	12
L-Tyr-OMe	0
D-Met-OMe	5
L-Met-OMe	0
D-Glu(OMe)-OMe	0
L-Glu(OMe)-OMe	33

Activity was expressed as a percentage of the activity obtained with D-Phe-OMe as the substrate. Other amino acid methyl esters, including D-/L-Ala, D-/L-Arg(NO_2), L-Cys(Bzl), Gly, D-/L-His, L-Ile, D-/L-Leu, D-/L-Lys(Z), D-/L-Pro, D-/L-Ser, D-/L-Thr, and D-/L-Val, were inert as substrates.

Based on the substrate specificity, peptide synthesis was examined using the aminoacyltransferase. As shown in TABLE 1.8, D-amino acid peptides whose chain lengths were from two to seven were synthesized by the aminoacyltransferase even in low yields. On the other hand, dipeptide was only synthesized when L-glutamate

methyl ester employed as substrate. Therefore, it was suggested that reaction mechanism of the aminoacyltransferase for L-glutamate methyl ester was different from that for methyl esters of D-amino acids. In addition to peptide synthesis from amino acid esters, the aminoacyltransferase recognized D,L-amino acid amides, and it was demonstrated that acetyl-D-phenylalanyl dipeptides could be sequentially synthesized from acetyl-D-phenylalanine methyl ester and D,L-amino acid amides. Although α-amino acid esterase mentioned above also showed sequential peptide synthesizing activity from methyl ester and amide of amino acid, the aminoacyltransferase catalyzed the sequential synthesis in higher yield. Therefore, it is thought that the aminoacyltransferase is the most useful enzyme for D-amino acid peptide synthesis, reported so far.

TABLE 1.8 Retention times, molecular masses, and proposed structures of the reaction products from amino acid methyl esters (7)

Substrate	Retention time (min)	Molecular mass (Da)	Yield (%)	Structure
D-Asp(OMe)-OMe	2.7	290.3		$(D\text{-Asp(OMe)})_2$-OMe
	3.1	419.4		$(D\text{-Asp(OMe)})_3$-OMe
D-Met-OMe	5.7	230.3		$(D\text{-Met})_2$-OMe
D-Phe-OMe	8.8	644.9	15	$(D\text{-Phe})_4$-OMe
	9.6	798.2	24	$(D\text{-Phe})_5$-OMe
	10.2	915.4	7.1	$(D\text{-Phe})_6$-OMe
D-Trp-OMe	8.1	809.2	2.1	$(D\text{-Trp})_4$-OMe
	8.9	1,003.40	1.6	$(D\text{-Trp})_5$-OMe
D-Tyr-OMe	6.3	1,216.60	0.9	$(D\text{-Tyr})_7$-OMe
L-Glu(OMe)-OMe	4.1	286.3		$(L\text{-Glu(OMe)})_2$-OMe

1.3. D-Alanine-D-alanine ligase with altered substrate specificity

1.3.1. Bacterial peptidoglycan

Bacterial peptidoglycan is localized in the cell wall and protects the cell from environmental stresses. Peptidoglycan is polymer of sugars and amino acids, and the basic unit of peptidoglycan consists of N-acetyl glucosamine, N-acetyl muramate, and pentapeptide (L-Ala-D-Glu-DAP or L-Lys-D-Ala-D-Ala) (8). Five enzymes, *i.e.* UDP-N-acetyl muramate-L-alanine ligase (MurC), UDP-N-acetylmuramoyl--L-alanine-D-glutamate ligase, (MurD) UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--L-lysine ligase (MurE), UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase (MurF), and D-alanine-D-alanine ligase (Ddl) catalyzes the biosynthesis of peptide chain in the basic unit (FIG. 1.1) (9-15).

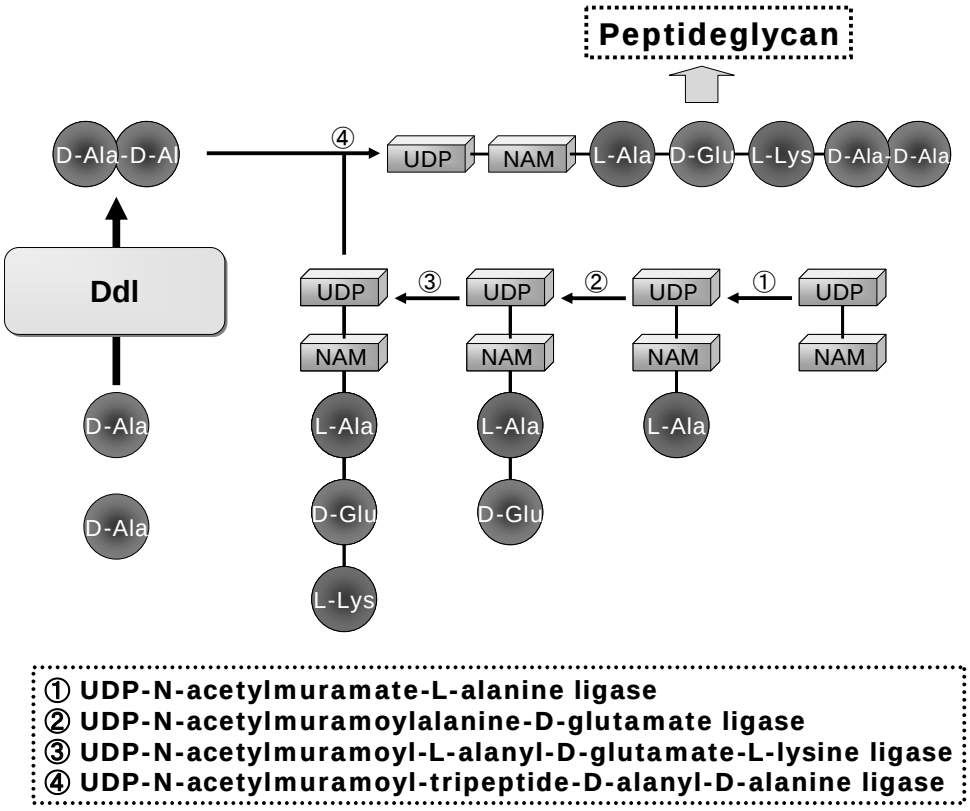

FIG. 1.1. Biosynthetic pathway of peptidoglycan.

Although MurC, D, E, and F synthesize or elongate the glycopeptides, Ddl synthesizes D-alanyl-D-alanine, one of D-amino acid dipeptide, from D-alanine. In the synthesis of D-alanyl-D-alanine with Ddl, free N-terminal D-alanine is activated by phosphorylation following hydrolysis of ATP, and forms phosphoester intermediate. Subsequently, the lone pair on nitrogen atom of C-terminal D-alanine attacks the phosphoester, and synthesized D-alanyl-D-alanine is released (FIG. 1.2) (16).

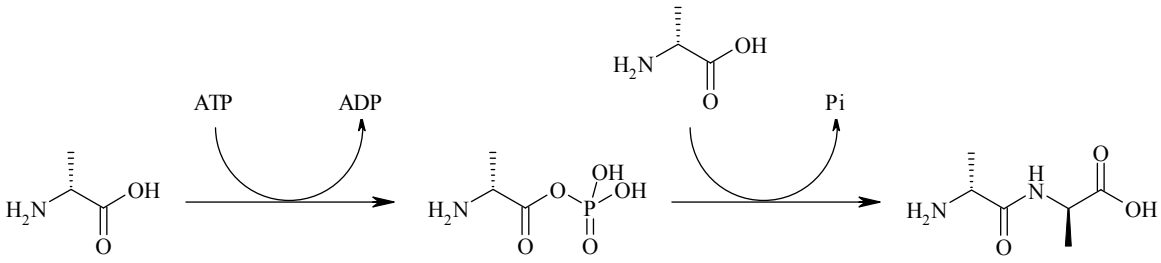

FIG. 1.2. D-Alanyl-D-alanine synthesis catalyzed by Ddl.

Of course, the substrate specificity of Ddl for D-alanine is defined by tertiary structure of Ddl, however, it was reported that some D-amino acids and related compounds with the lone pair on functional group bound to α-carbon atom were recognized as substrate by Ddl, as mentioned below.

1.3.2. Ddl from vancomycin-resistant *enteroccossi*

Ddls with altered substrate specificity were found from vancomycin-resistant *enteroccossi* (VRE) (17). Vancomycin is known as the effective antibiotics against gram-positive bacteria. In these bacteria, vancomycin binds to D-alanyl-D-alanine terminus of the basic unit of peptidoglycan, and inhibits the formation of cross-linking between peptidoglycan intermediates (FIG. 1.3). Therefore, D-alanyl-D-alanine terminus is important structure for vancomycin binding. In contrast to vancomycin-sensitive bacteria that have D-alanyl-D-alanine terminus, there are some

vancomycin-resistant *enteroccossi* that have D-alanyl-D-lactate or D-alanyl-D-serine terminus. VREs become resistant against vancomycin because vancomycin shows lower affinity for these terminus than that for D-alanyl-D-alanine in VREs (18-22). As mentioned above, the synthesis of D-alanyl-D-alanine is catalyzed by Ddl, therefore it was suggested that VRE had mutant Ddl with altered substrate specificity.

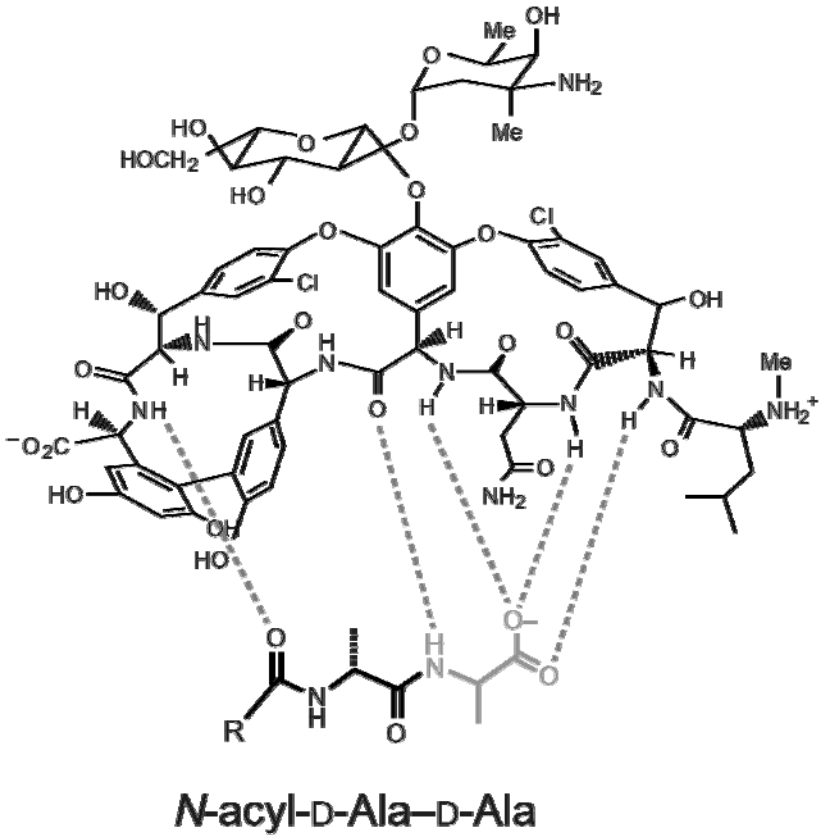

FIG. 1.3. Binding of vancomycin to D-alanyl-D-alanine peptidoglycan termini. From reference 17.

First example of Ddl with altered substrate specificity was reported by Bugg *et al.* (23). The Ddl was purified from *Enterococcus faecium* BM4147 that was resistant against vancomycin, and the purified Ddl recognized D-methionine and D-phenylalanine as C-terminal substrate. Subsequently, it was revealed that the Ddl named VanA

catalyzed the formation of ester bond between D-alanine and D-lactate, and synthesized D-alanyl-D-lactate (24). Thereby, it was confirmed that VanA ligase partially maintained the vancomycin-resistant mechanism of VREs. After the discovery of VanA ligase, VanB and VanC ligase that showed substrate specificity for D-lactate and D-serine were discovered from *En. faecalis* V583 and *En. casseliflavus* ATCC 25788 (25, 26). Although both VanA and VanB ligase synthesize D-alanyl-D-lactate, the two ligases are distinguished by VanA or VanB phenotype of host strains. VanA phenotype strain is resistant to vancomycin and teicoplanin. On the other hand, VanB phenotype strain is resistant to vancomycin but sensitive to teicoplanine. The studies based on molecular evolution revealed that these ligases existed on independent group in phylogenetic tree (17).

1.3.3. Structure based analysis of Ddl and Van ligases

The crystal structure of DdlB from *Escherichia coli* (EcDdlB) complexed with ADP and a phosphinophosphate transition-state analog have been resolved, and several active-site residues were identified by Fan *et al.* (27). Subsequently, Shi *et al.*. mutated several of these amino acids including a trio of residues, Y216, S150, and E15, that form a hydrogen-bonded network (28). The hydrogen bonds of this triad connect a mobile ω-loop containing Y216, via S150, and E15 to the binding of the α-amino group of the first molecule of substrate D-alanine as it undergoes activation as the acyl phosphate D-alanyl phosphate. All the three mutants that interrupt or modulate the hydrogen bond network, Y216F, S150A, and E15Q of EcDdlB, retained significant Ddl activity with *k*cat values ranging from 38% to 110% of wild type, although *K*m values for both the first and second D-alanine substrates were elevated 1-2 orders of magnitude from the wild-type *K*m values of 1.2 μM (N-terminal D-alanine) and 1.1 mM

(C-terminal D-alanine). Park *et al.* analyzed these three EcDdlB mutants further and found that two of the mutants, Y216F and S150A, have gained VanA-type activity, the ability to make significant quantities of depsipeptide products D-alanyl-D-lactate and D-alanyl-D-hydroxybutyrate (TABLE 1.9) (16). Moreover, E15Q mutant EcDdlB showed novel ligase activity that synthesize D-lacyl-D-alanine. Therefore, it was suggested that only one amino acid substitution could convert EcDdl to VanA ligase.

TABLE 1.9 Kinetic Parameters of EcDdlB and VanA ligase (16)

	Activity	pH	K_{m_2} for C-terminal D-alanine or D-lactate (mM)	k_{cat} (min^{-1})	k_{cat}/K_{m_2} (mM^{-1} min^{-1})
EcDdlB	A-A[a]	6.0	8.0	710	89
		7.5	2.0	880	440
		9.2	1.1	1600	1500
S150A	A-A	6.0	140	200	1.4
		7.5	80	980	12
		9.2	44	1100	26
	A-L[b]	6.0	1.6	1.4	0.87
		7.5	2.2	3.4	1.5
	A-H[c]	6.0	5.3	1.1	0.21
Y216	A-A	6.0	150	450	3
		7.5	16	630	39
		9.2	4.8	1200	260
	A-L	6.0	5.1	7.8	1.5
		7.5	27	42	1.6
	A-H	6.0	8.9	5.3	0.6
		7.5	40	6.2	0.16
E15Q	A-A	6.0	82	200	2.4
		7.5	74	820	11
		9.2	65	1100	17

[a] D-Alanine-D-alanine ligase. [b] D-Alanine-D-lactate ligase.
[c] D-Alanine-D-hydroxybutylate ligase.

Amino acid sequence alignment of EcDdlB and VanC ligase revealed that almost all the active site residues of EcDdlB were conserved in VanC ligase, but Y210 and L282 active site residues of EcDdlB were substituted to F250 and R322 in VanC ligase (29). Healy *et al.* expected that F250 and R322 would concern to the altered substrate specificity of VanC ligase, and mutated these residues. As shown in TABLE 1.10, F250Y and R322M mutants showed less selectivity toward D-serine, and the substrate selectivity of F250Y/R322M double mutant was totally converted to D-alanine. Therefore, conversion of VanC ligase to EcDdl was also achieved by one or two amino acid substitutions, suggesting substrate specificity of variety of Ddls would be changed by a few numbers of amino acid substitution.

TABLE 1.10 VanC mutants with reversed specificity for D-Ala-D-Ala mutant ligases (29)

Enzyme	$k\,\mathrm{cat}/K\,\mathrm{m}_2$ (D-Alanine) / $k\,\mathrm{cat}/K\,\mathrm{m}_2$ (D-Serine)	Swich in specificity (fold)
VanC ligase	0.0042	1
F250Y	0.19	44
R322M	0.15	36
F250Y/R322M	26	6,200

1.4. Objective of this thesis

As mentioned in sector 1.2, D-amino acid esters or amides are necessary as substrate for D-amino acid peptide synthesis utilizing peptidases, esterases, and aminoacyltransferase reported so far. Thereby, derivatization of free D-amino acids is needed in the production processes utilizing these enzymes. Besides, the reaction conditions of theses enzymes, excluding aminoacyltransferase, need addition of organic solvent to prevent peptide hydrolyzing activity. Due to the hydrolyzing activity, the

maximum synthetic yield of D-amino acid peptide was limited to 60%. Therefore, it is considered that effective production of D-amino acid peptide could be achieved with enzyme that utilize free D-amino acid as substrate and have no hydrolyzing activity.

Then, the author focused on D-alanine-D-alanie ligase as a useful enzyme for the production of D-amino acid peptide, especially D-amino acid dipeptide. Ddl utilizes free D-alanine as substrate and synthesize D-alanyl-D-alanine without reverse reaction, therefore it was expected that Ddl would overcome the main problems on D-amino acid peptide synthesis reported so far. However, substrate specificity of Ddl was restricted and large amount of ATP was necessary for D-amino acid dipeptide synthesis by Ddl. In this thesis, Ddls with broad substrate specificity were explored based on diversity of Ddl from various bacteria, and effective D-amino acid dipeptide synthesis utilizing Ddl coupled with amino acid racemase or ATP regeneration systems was performed to develop the effective production process of various D-amino acid dipeptides.

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Chapter 2

D-Amino Acid Dipeptide Production utilizing D-Alanine-D-Alanine Ligases with Novel Substrate Specificity

2.1. Introduction

Peptides were discovered as antimicrobial compounds and signal molecules in nature, and are utilized as functional foods and drug materials. Although there are few natural peptides that contain D-amino acids, peptides incorporating D-amino acids (D-amino acid peptides) are expected to increase the functional variety and applications of peptides (1-5).

D-Amino acid peptides have been produced by organic synthesis (6, 7) and enzymatic synthesis using the reverse reaction of peptidase (8, 9), esterase (10) or aminoacyltransferase (11). However, the complicated reaction steps and the racemization of amino acids are difficulties associated with organic synthesis. In enzymatic synthesis, expensive materials such as D-amino acid ester are required, and the yield of synthesized D-amino acid peptide is limited to 60% (8). Therefore, the establishment of a more effective production system for D-amino acid peptides is desired.

Thus, D-alanine-D-alanine ligase (Ddl) associated with the synthesis of bacterial peptidoglycan was examined as a useful enzyme for the effective production of various D-amino acid peptides. A mutant Ddl from vancomycin-resistant enterococci (VREs) recognizes D-serine as a substrate in addition to D-alanine (12). Therefore, Ddls from

several other bacteria are expected to show broad substrate specificities on the basis of bacterial diversity. Since Ddl synthesizes D-alanyl-D-alanine from free D-alanine by a one-step reaction, it is expected that various D-amino acid dipeptides could be produced effectively using Ddls with broad substrate specificities. Moreover, Ddls selectively synthesize D-alanyl-D-alanine from D,L-alanine, and this chiral selectivity is effective for the high chirality production of D-amino acid dipeptides from D,L-amino acid racemates or the chiral resolution of D,L-amino acids.

In this chapter, the genes encoding Ddls from *E. coli* K 12, *O. iheyensis* JCM 11309, *Synechocystis* sp. PCC 6803 and *T. maritima* ATCC 43589, which showed diversity in their primary structures (Fig. 2.1), were cloned, and the substrate specificities of these recombinant Ddls were investigated. In addition, several D-amino acid homo-dipeptides were produced using EcDdlB.

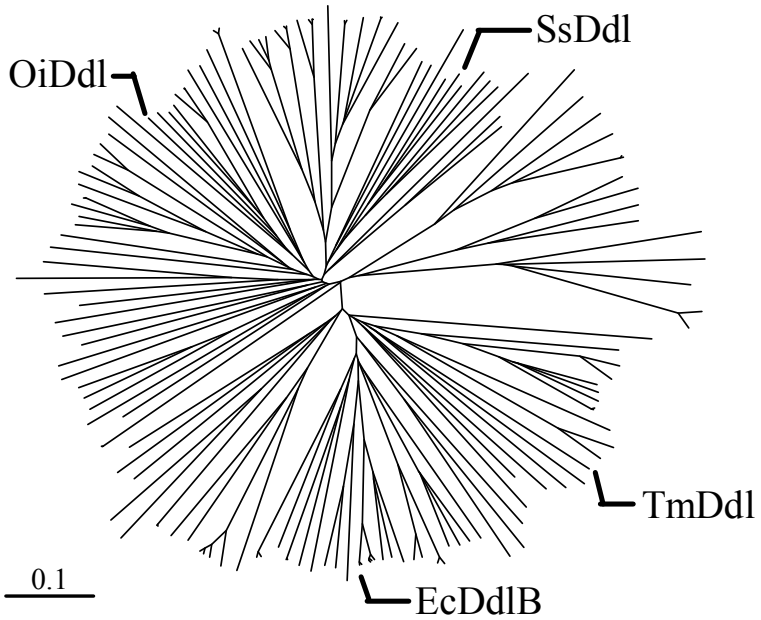

FIG. 2.1. Phylogenetic tree of Ddls.

2.2. Materials and methods

2.2.1. Bacterial strains and culture conditions

E. coli JM 109 and *E. coli* BL 21(DE3) were purchased from Nippon Gene (Tokyo) and Merck (San Diego, CA, USA), respectively. *O. iheyensis* JCM 11309 was purchased from RIKEN (Saitama). Genomic DNA of *T. maritima* ATCC 43589 was purchased from the American Type Culture Collection and that of *Synechocystis* sp. PCC 6803 was provided by the Kazusa DNA Research Institute.

E. coli strains were grown at 37^{o}C in Luria-Bertani medium, and *O. iheyensis* JCM 11309 was grown at 30^{o}C in modified Horikosi-I medium consisting of 1% glucose, 0.5% peptone, 0.5% yeast extract, 1% NaCl, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 1% $NaHCO_3$.

2.2.2. Chemicals and materials

D-Amino acids and D-alanyl-D-alanine were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade and obtained from commercial sources.

2.2.3. Gene cloning

Genomic DNAs of *E. coli* K 12 and *O. iheyensis* JCM 11309 were prepared from cultured cells using DNeasy Tissue kit (Qiagen, Valencia, CA, USA). *Ddl*s were amplified by PCR with KOD-PLUS DNA polymerase (Toyobo, Osaka) in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) using the primers 5'-GGTATTGAGGGTCGCATGACTGATAAAATCGCG-3' and 5'-AGAGGAGAGTT--AGAGCCTTAGTCCGCCAGT-3' for EcDdlB, 5'-GGTATTGAGGGTCGCATGAAG-

-AAAAAAGTTGG-3’ and 5’-AGAGGAGAGTTAGAGCCCTATCCATCACTT-3’ for OiDdl, 5’-GGTATTGAGGGTCGCATGCGTGTGGGGC-3’ and 5’-AGAGGAGAGTT--AGAGCCTTATTGGTGACCGGGA-3’ for SsDdl and 5’-GGTATTGAGGGTCGCAT--GAGAGTGGCTCTC-3’ and 5’-AGAGGAGAGTTAGAGCCTCATACTCTCACCCC--TT-3’ for TmDdl.

Amplified *ddl*s were treated with T4 DNA polymerase and introduced into the pET 30 Xa/LIC vector (Merck) as instructed in the pET 30 Xa/LIC vector kit manual, and *E. coli* JM 109 cells were transformed. After plasmid isolation, the recombinant plasmids obtained were introduced into *E. coli* BL21(DE3).

2.2.4. Overexpression of *ddl*s and enzyme purification

Each recombinant *E. coli* BL 21(DE3) strain harboring a recombinant plasmid carrying *ddl*s was cultured for 16 h in 100 ml of Luria-Bertani medium with 20 μg/ml kanamycin and 0.1 mM IPTG. Cells were harvested by centrifugation at 3000×*g* for 10 min. The cell pellet was treated with 5 ml of BugBuster Protein Extraction reagent (Merck) for 20 min, then centrifuged at 16,000×*g* for 30 min, and the supernatant was collected as cell-free extract.

The cell-free extract was applied onto a HisTrap HP 1-ml column (Amersham Bioscience, Piscataway, NJ, USA) equilibrated with 100 mM Tris-HCl (pH 8.0), 500 mM NaCl and 40 mM imidazole, and the column was washed with 10 column volumes of the same buffer. The His-tagged protein was eluted with 100 mM Tris-HCl (pH 8.0), 500 mM NaCl and 300 mM imidazole and desalted on a HiTrap Desalting 5-ml column (Amersham Bioscience) equilibrated with 100 mM Tris-HCl (pH 8.0). The desalted protein was used as purified Ddl.

2.2.5. Ddl activity assay by released phosphate

Ddl activity was measured by determining the amount of released phosphate on the basis of the method of Daub *et al.* (13). The reaction mixture for the Ddl activity assay, containing 100 μg/ml purified Ddl, 25 mM D-amino acid, 12.5 mM ATP and 12.5 mM $MgSO_4$, was adjusted to pH 8.0 and the reaction was carried out at 37°C. The activity was determined by released phosphate colorimetry using Determinar L IP (Kyowa Medex, Tokyo) by measuring the absorbance at 595 nm.

2.2.6. Determination of D-amino acid dipeptides

The structure and chirality of D-alanyl-D-alanine were determined by HPLC using a WH-C18A (3 μm) column (Hitachi High Technologies, Tokyo). A gradient elution of solvent A [50 mM KH_2PO_4 (pH 2.7 by phosphate):acetonitrile:methanol=18:1:1] and solvent B [50 mM KH_2PO_4 (pH 2.7 by phosphate):acetonitrile:methanol=12:7:1] was used with the following program: 0 to 24 min; a linear increase in solvent B to A:B of 55:45, held to 30 min, 30 to 50 min; a linear increase to A:B of 0:100, held to 55 min. After each gradient elution, the column was washed with solvent C [water: tetrahydrofuran; acetonitrile=1:1:3] for 7 min and re-equibrated with solvent A for 20 min. The flow rate was maintained at 0.5 ml / min, and the column temperature was kept at 40°C. After separation, UV absorption was monitored at 340 nm. D-Alanyl-D-alanine and D-amino acids were derivetized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide and subjected to HPLC.

Molecular mass of peptide was determined using MALDI-TOF MS analysis by REFLEX (Bruker Daltonics, Billerica, MA, USA) and 2,5-dihydroxy benzoic acid was used as matrix. NMR analysis was performed with a DMX500 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany).

2.2.7. Production of D-amino acid dipeptide

D-Amino acid dipeptides were prepared in a reaction mixture containing 250 μg/ml purified Ddl, 40 mM D-amino acid, 20 mM ATP and 20 mM $MgSO_4$ at 37°C for 7 h, and the amount of dipeptide produced was determined by released phosphate colorimetry.

2.3. Results

2.3.1. Overexpression of recombinant *ddl*s and enzyme purification

Four recombinants were obtained by the transformation of *E. coli* BL21(DE3) with the recombinant plasmids pEcDdlB, pOiDdl, pSsDdl and pTmDdl; which harbored the genes encoding *ddl*s from *E. coli* K 12, *O. iheyensis* JCM 11309, *Synechocystis* sp. PCC 6803 and *T. maritima* ATCC 43589, respectively.

As shown in Fig. 2.2, all *ddl*s were expressed in the soluble fraction after cultivation of each recombinant *E. coli* strain and Ddls were purified to homogeneity by Ni^{2+} affinity chromatography. The specific activities of the purified EcDdl, OiDdl, SsDdl and TmDdl were 2.9, 0.4, 1.6 and 1.3 μmol/min/mg, respectively.

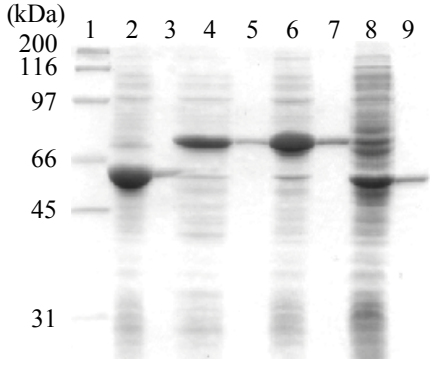

FIG. 2.2. Purification of His-tagged Ddls from recombinant *E. coli*. Lanes: 1, molecular mass standards; 2, 3, EcDdl; 4, 5, OiDdl; 6, 7, SsDdl; 8, 9, TmDdl. Lanes: 2, 4, 6, 8 indicate cell-free extracts from each recombinant *E. coli* and 3, 5, 7, 9 indicate purified Ddls after Ni^{2+} affinity chromatography.

2.3.2. Substrate specificity of Ddls

D-Amino acid dipeptide synthesis using Ddl requires the hydrolysis of ATP. The substrate specificities of Ddls for twenty D-amino acids including glycine were investigated by determination of the concentration of released phosphate.

Released phosphate was detected in the reaction mixtures containing D-alanine, D-serine, D-threonine, D-cysteine or glycine, with EcDdlB, SsDdl or TmDdl. However, phosphate was released only from the reaction mixture containing D-alanine with OiDdl (Fig. 2.3).

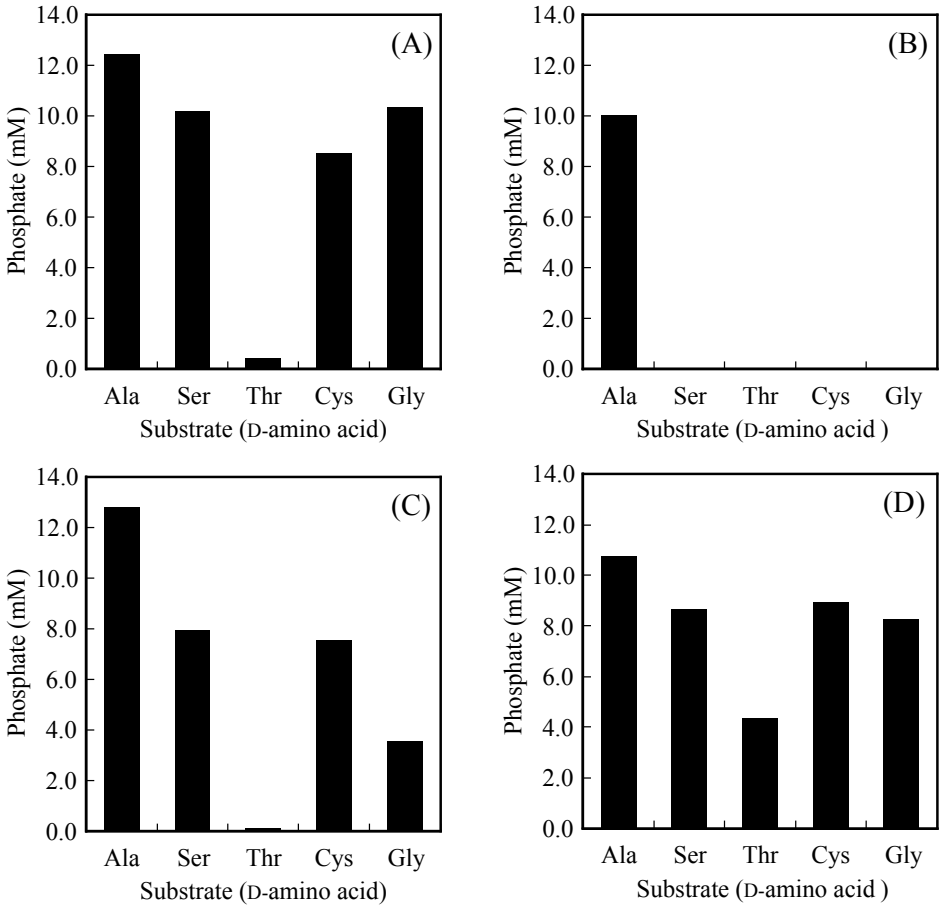

FIG. 2.3. Substrate specificities of Ddls. The composition of the reaction mixture was described in Materials and methods. After incubation at 37ºC for 20 h, the concentration of phosphate released was determined. (A) EcDdlB; (B) OiDdl; (C) SsDdl; (D) TmDdl.

2.3.3. Structural determination and quantification of products

The structures of the products of the reaction mixtures that resulted in phosphate release was determined by HPLC, NMR, and MALDI-TOF MS. For D-alanyl-D-alanine, its structure and chirarity were determined by HPLC. As shown in Table 2, it was confirmed that the amounts of D-alanyl-D-alanine determined by HPLC and the released phosphate assay were almost equal. Therefore, the quantification of D-amino acid dipeptides was only determined by released phosphate in the subsequent experiments. Furthermore, structure was determined for the products from reaction mixtures containing D-serine, D-threonine, D-cysteine or glycine by NMR and MALDI-TOF MS. As a result, signals and peaks corresponding to the molecular structure and mass of D-seryl-D-serine, D-threonyl-D-threonine, D-cysteinyl-D-cysteine and glycyl-glycine were detected, while no trimers or tetramers were detected (data not shown). Then, it was confirmed that EcDdlB, SsDdl and TmDdl have broad substrate specificities for D-alanine, D-serine, D-threonine, D-cysteine and glycine; D-amino acid homo-dipeptides were synthesized. On the other hand, it was also confirmed that OiDdl has a high substrate specificity for D-alanine, and only D-alanyl-D-alanine was synthesized. In addition, it was confirmed by qualitative HPLC that EcDdlB synthesizes D-alanyl-D-serine with D-alanyl-D-alanine and D-seryl-D-serine as by-products from a D-alanine and D-serine mixture. However, D-seryl-D-alanine was not detected.

2.3.4. Production of various D-amino acid dipeptides using EcDdlB

Since EcDdlB, SsDdl and TmDdl showed broad substrate specificities, the production rate of D-amino acid dipeptides using these Ddls was investigated by quantification of D-alanyl-D-alanine produced in a short reaction time.

As shown in Table 2.1, the reaction mixture containing EcDdlB showed the highest production of D-alanyl-D-alanine, with 5.3 mM D-alanyl-D-alanine being produced from 25 mM D-alanine in 20 min. Therefore, the production of D-amino acid homo-dipeptides using EcDdlB was investigated in detail.

TABLE. 2.1 D-Alanyl-D-alanine productivity

Ddl	D-Alanyl-D-alanine (mM)	
	HPLC assay	Phosphate assay
EcDdlB	5.7	5.3
OiDdl	0.7	0.6
SsDdl	3.2	2.9
TmDdl	2.6	2.3

The production of various D-amino acid dipeptides from free D-amino acids using EcDdlB was investigated. In the reaction at 37°C, although the yield of D-threonyl-D-threonine was only 2%, the yields of D-alanyl-D-alanine, D-seryl-D-serine, D-cysteinyl-D-cysteine and glycyl-glycine were up to 71%, 71%, 60% and 77%, respectively (Fig. 2.4).

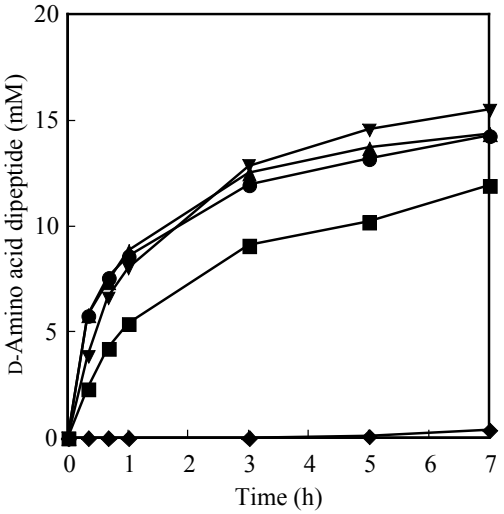

FIG. 2.4. Production of D-amino acid dipeptides using EcDdlB. Reaction mixtures containing D-alanine, D-serine, D-threonine, D-cysteine and glycine were incubated at 37°C. The concentration of phosphate released was detected at each reaction. Symbols: circles, D-alanyl-D-alanine; triangles, D-seryl-D-serine; diamonds, D-threonyl-D-threonine; squares, D-cysteinyl-D-cysteine; upside-down triangles, glycyl-glycine.

2.4. Discussion

It was previously reported that some D-amino acids can be introduced at the C-terminus of synthesized D-amino acid dipeptides by Ddls from VREs or *E. coli* (14). On the other hands, it was clarified that other D-amino acids, in addition to D-alanine, could be introduced into the N-terminus of synthesized D-amino acid dipeptides by Ddls from non-VRE bacteria, such as *E. coli* K12, *Synechocystis* sp. PCC 6803, and *T. maritima* ATCC 43589, in this study. While these three Ddls showed the broad substrate specificity, OiDdl showed the strict substrate specificity for D-alanine. Thus, it was revealed that there are two types of Ddls, one with a broad substrate specificity and the other with a high substrate specificity for D-alanine. The difference in substrate specificity between EcDdlB, SsDdl, TmDdl and OiDdl leads to the determination of specific amino acid residues of Ddl that are involved in the recognition of substrate, by comparing multiple alignments and the secondary or tertiary structures of these four Ddls. The multiple alignments of the four Ddls are shown in Fig. 2.5, and the residues that interact with D-alanine, ATP and Mg^{2+} in EcDdlB (15, 16) are indicated. There were several residues of OiDdl that differed from the conserved residue of EcDdlB, SsDdl and TmDdl at the same position; and D141, Y158 and S254 were considered particularly important residues involved in the high substrate specificity of OiDdl, because the properties of these residues were quite different from those of the conserved residues. Furthermore, D141 was adjacent to K140, which corresponds to the substrate-interacting residue in EcDdlB, and S254 was located in the highly conserved region referred to as the ω-loop in EcDdlB, which is considered an important region involved in the formation of the active center. Therefore, these residues should have some effect on substrate specificity and the substitution of these

residues is expected to expand the substrate specificity of OiDdl. These studies provide some critical observations concerning the mechanism of the vancomycin resistance of VREs, and indicate that it is possible to control the substrate specificity of Ddls.

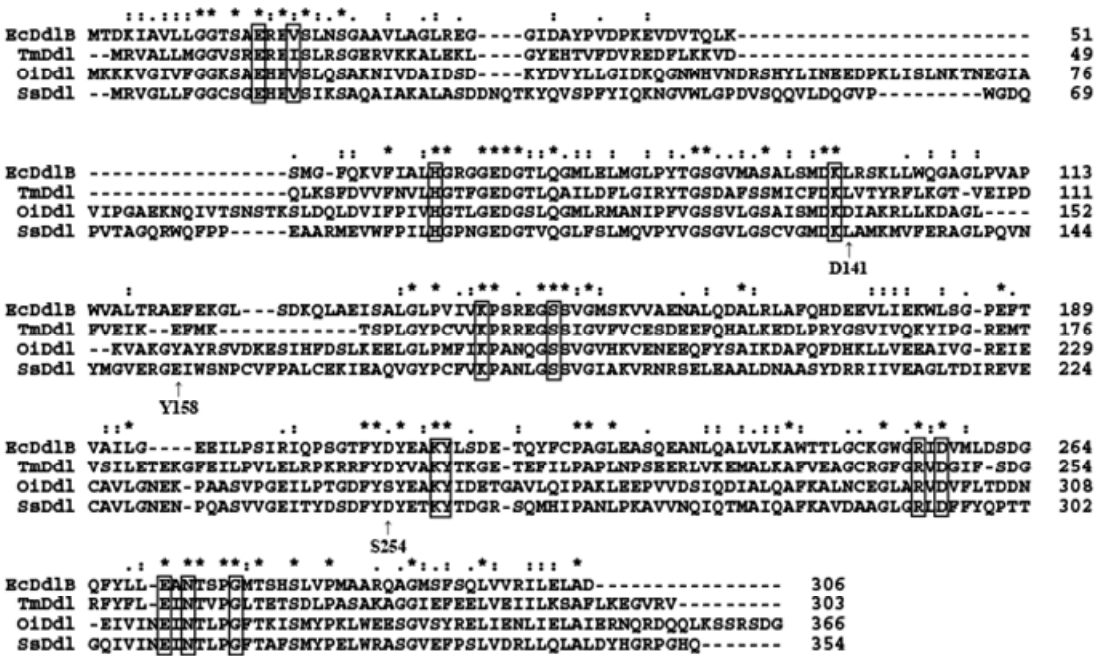

FIG. 2.5. Multiple alignments of Ddls. Multiple alignments were calculated by ClustalX 1.83 and the residues that interact with D-alanine, ATP and Mg^{2+} in EcDdlB are enclosed. The asterisks indicate the amino acids conserved in EcDdlB, OiDdl, SsDdl and TmDdl.

In addition, the sequential selectivity was observed in the dipeptide synthesis from D-alanine and D-serine. This sequential selectivity was due to the affinity of the substrate to the first or second substrate binding site. This result indicates the possibility of the sequential synthesis of D-amino acid dipeptides by Ddls with a particular substrate specificity.

In the production of D-amino acid dipeptide utilizing EcDdlB, four kinds of D-amino acid dipeptides could be produced in the high yield. These high yields could be achieved because there was no reverse reaction during the process using Ddl and the

D-amino acid dipeptides produced were not hydrolyzed. This is one of the advantages of the process using Ddl. In the production of D-amino acid peptides using peptidase or esterase, the hydrolysis of the dipeptide produced would be the cause of the low yield. Therefore, strict control of hydrolysis was necessary. Moreover, in the process using Ddl, D-amino acid dipeptides were produced from free D-amino acids in a one-step reaction. The direct production of D-amino acid dipeptides from free D-amino acids is another advantage of this process.

On the other hand, there are two major problems in the production process using Ddl. One is the inhibitory effect of the dipeptide produced (17). As shown in Fig. 6, it was considered that the decrease in the production rate was caused by this inhibitory effect. The other problem is that ATP is necessary for the reaction of Ddl. From the viewpoint of production cost, the requirement for ATP is the major disadvantage of this process. However, several ATP regeneration systems have been reported (18-20), and the coupling reaction system of Ddl and ATP regeneration is considered a model of a non-ATP-requiring production process for D-amino acid dipeptides. Furthermore, D-amino acid dipeptides are comparatively stable in bacterial cells, therefore their production using Ddl is expected to be applied to fermentation.

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Chapter 3

D-Amino Acid Dipeptide Production from L-Amino Acid Utilizing a Coupling Reaction of D-Alanine-D-Alanine Ligase and Amino Acid Racemase

3.1. Introduction

Peptides containing D-amino acids (D-amino acid peptides) are expected to enlarge the functional variety of peptides as they have more kinds of constitutive residues than natural peptides. Previously, many efforts for enzymatic synthesis of D-amino acid peptides were carried out utilizing mainly reverse reaction of peptidase and esterase (1-7). However, these enzymes need D-amino acid esters as substrate and difficult settings of reaction conditions to prevent their hydrolyzing activity. Then the author focused on D-alanine-D-alanine ligase (Ddl) as a useful enzyme for effective production of D-amino acid peptides. Ddl catalyzes the ligation of free D-alanine and synthesizes D-alanyl-D-alanine as precursor of peptidoglycan in bacterial cell; therefore, free amino acid can be utilized for peptide synthesis with Ddl. Besides, the reverse reaction, *i.e.* degradation of synthesized peptide, would not occur by Ddl because the reaction of Ddl progresses following ATP hydrolysis. Although it was considered that Ddl recognized only D-alanine as substrate, Ddls with broad substrate specificity were discovered through the evaluation of specificity of Ddls from various bacteria (8). Especially, Ddl from *Escherichia coli* K-12 (EcDdlB) showed the broad substrate specificity toward D-alanine, D-serine, D-cysteine, D-threonine, and glycin, and synthesized corresponding homo-dipeptides. However, two major problems are still

remained for an effective production of D-amino acid dipeptides.

One of the problems is that EcDdlB can only utilize expensive D-amino acid, and is that ATP consumed in the reaction with EcDdl is also an expensive material and is required equal molar to synthesized D-amino acid dipeptide. To improve the first problem, utilization of broad specificity amino acid racemase from *Pseudomonas putida* IFO 12996 (BAR) was focused as a useful enzyme to supply D-amino acid from inexpensive L-amino acid. It was reported that BAR showed broad substrate specificity toward basic and neutral amino acid, and was already purified partially (9-15). Besides, the amino acid sequence around active site was reported, but total sequence of the gene encoding BAR was not revealed. Then, the author started from cloning of the gene, and coupling reaction with EcDdlB and the cloned BAR was demonstrated. Moreover, the reaction was applied into the resting cell reaction to utilize the ability for ATP regeneration of the cell for effective supply of ATP to the reaction.

3.2. Materials and methods

3.2.1. Bacterial strains, plasmids, and culture conditions

P. putida IFO 12996 and *Escherichia coli* BL21 (DE3) / pLysS were purchased from the Institute of Fermentation Osaka (Osaka, Japan) and Merck KGaA (Darmstadt, Germany), respectively. *E. coli* JM 109 was supplied from Nippon Gene (Tokyo, Japan). Plasmid vector pET 21a (+), pET 30 Xa/LIC, and pTrcHis2A were from Merck KGaA and Invitrogen (California, USA). *E. coli* BL21 (DE3) / pEcDdlB that produce His×6-tagged EcDdlB was prepared as described previously (8).

P. putida IFO 12996 and *E. coli* strains were grown in LB medium at 37°C, with

120 rpm rotary shaking for 16 h. In the case of gene expression, recombinant *E. coli* was grown in LB medium containing appropriate antibiotics (50 μg/ml ampicillin, 25 μg/ml kanamycin, or 25 μg/ml chloramphenicol), and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C, with 120 rpm rotary shaking for 16 h.

3.2.2. Chemicals and materials

D-Amino acids were purchased from Kanto Kagaku (Tokyo, Japan) or Kokusan Kagaku (Tokyo, Japan). 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide was from Sigma-Aldrich (St. Louis, MO, USA). His-Trap HP 1-ml column, PD-10 column, and ÄKTA explorer 10S were from Amersham Bioscience (NJ, USA). Model 550 microplate reader was purchased from Bio-Rad Laboratories (CA, USA). L-7000 series high-performance liquid chromatography (HPLC)-equipped WH-C18A (3 μm) column was from Hitachi High-Technologies (Tokyo, Japan). All other chemicals were of reagent grade and obtained from commercial sources.

3.2.3. Gene cloning

The gene encoding BAR was amplified by polymerase chain reaction using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) with *Nde*I and *Eco*RI restriction sites at upstream and downstream of bar, respectively. After the digestion by *Nde*I and *Eco*RI, amplified *bar* was ligated to pET 21a (+) vector using DNA Ligation kit version 2.1 (Takara Bio) and introduced into *E. coli* JM 109 for plasmid isolation. The isolated recombinant plasmid harboring *bar* was introduced into *E. coli* BL 21(DE3)/pLysS. His×6 tagged BAR was purified from the resulting recombinant *E. coli*.

In the construction of EcDdlB and BAR coexpression plasmid, the genes

encoding EcDdlB and BAR were amplified with *Nco*I and *Bgl*II restriction site at upstream and downstream of each gene. The each amplified PCR product was digested by *Nco*I and *Bgl*II, and ligated to pTrcHis2A vector. Using resulting plasmid, *ecddlB* including Trc promoter and terminator was amplified with *Nur*I restriction site at both 5' and 3' ends, and ligated at *Nur*I site of pTrcHis2A harboring *bar*. The resulting plasmid was introduced into *E. coli* JM 109, and the recombinant *E. coli* was used in the resting cell reaction.

3.2.4. Purification procedure of His×6-tagged protein

The recombinant protein with His×6 tag was produced in recombinant *E. coli*. The cells were harvested at 3,000×*g* for 10 min, and the cell pellet was treated with 5 ml of BugBuster Protein Extraction Reagent (Merck) for 20 min, then centrifuged at 16,000×*g* for 30 min, and the supernatant was collected as cell-free extract.

The cell-free extract was applied onto a His-Trap HP 1-ml column equilibrated with 100 mm Tris-HCl (pH 8), 150 mM NaCl, and 10 mM imidazole, and the column was washed with 10 column volumes of the same buffer. The His×6-tagged protein was eluted by linear gradient with 100 mM Tris-HCl (pH 8), 150 mM NaCl, and 500 mM imidazole and desalted on a PD-10 column equilibrated with 100 mM Tris-HCl (pH 8.5).

3.2.5. BAR activity assay

The reaction mixture containing 100 μg / ml His×6 tagged BAR and L- or D-amino acid was incubated at 40°C. In BAR activity assay, 50 mM (final conc.) L-form of lysine, arginine, histidine, alanine, serine, threonine, asparagine, glutamine, leucine, valine, isoleucine, methionine, phenylalanine, 20 mM L-form of tryptophan,

aspartate, glutamate or 1 mM L-form of tyrosine were added to the reaction mixture. The reaction was terminated by addition of equal amout of acetonitrile to the reaction mixture, subsequently synthesized D-amino acid was measured by HPLC.

3.2.6. D-Amino acid dipeptide production in the coupling reaction of purified EcDdlB and BAR

D-Amino acid dipeptides were produced in a reaction mixture containing 125 μg/ml each purified Ddl and BAR, 40 mM L-amino acid, 20 mM ATP and 20 mM $MgSO_4$ at 37°C for 7 h, and the amount of dipeptide produced was determined by released phosphate colorimetry using Determinar L IP (Kyowa Medex, Tokyo) by measuring the absorbance at 595 nm.

3.2.7. Resting cell reaction

Recombinant *E. coli* harboring coexpression plasmid was inoculated into 100 ml Luria-Bertani media containing 50 μg/ml ampicillin, and pre-cultured at 30ºC for 16 h with 150 rpm rotary shaking. The culture broth (65 ml) was added to 585 ml of terrific broth, and the recombinant *E. coli* was grown at 30ºC with aeration ratio at 650 ml/min in jar-fermentor. After 5.5 h cultivation, 0.1 mM IPTG (final conc.) was added to the broth and the gene expression was induced for one hour.

The resting cell reaction was performed as substrate solution containing 400 mM L-alanine and 200 g/l glucose was mixed with 450 ml of the cultured broth. During the reaction, pH was kept at 7.0 with 5 N NaOH, and 200 g/l glucose was supplied at 2 and 11 h reaction times. Produced D-alanyl-D-alanine and D,L-alanine was measured by HPLC.

3.2.8. HPLC analysis

Amino acids and D-alanyl-D-alanine were derivetized with FDAA and measured by HPLC equipped with WH-C18A column. A sample (100 μl) was mixed with 50 μl of 0.5% (w/v) FDAA solution in acetone and 40 μl of 0.5 M $NaHCO_3$ and incubated at 40ºC for 60 min. Afeter the deriverization, 40 μl of 0.1 N HCl was added to terminate the reaction and the FDAA derivetized compounds were subjected to the column. A gradient elution of solvent A [50 mM KH_2PO_4 (pH 2.7 by phosphate): acetonitrile: methanol=18:1:1] and solvent B [50 mM KH_2PO_4 (pH 2.7 by phosphate): acetonitrile: methanol=12:7:1] was used with the following program: 0 to 24 min; a linear increase in solvent B to A: B of 55: 45, held to 30 min, 30 to 50 min; a linear increase to A: B of 0: 100, held to 55 min. After each gradient elution, the column was washed with solvent C [water: tetrahydrofuran: acetonitrile=1:1:3] for 2 min and re-equibrated with solvent A for 20 min. The flow rate was maintained at 0.5 ml / min, and the column temperature was kept at 40°C. After separation, UV absorption was monitored at 340 nm.

3.3. Results

3.3.1. Cloning of the gene encoding BAR

The amino acid sequence near the active center of BAR has been determined as LTAVLKADAYGXGIGL in previous study (14). Then, the regions showing homologies to the amino acid sequence were searched in the complete genome sequence of *P. putida* KT2440 whose genome was disclosed in the National Center for Biotechnology Information, and three homologous regions were picked up. The ORF 1, ORF 2, and ORF 3 contained the homologous regions of LCAVLKADAYGHGIG

(14 identical residues), AVIKADAYGHG (nine identical residues), YGLGIGL (six identical residues), respectively, and the homologies of the three ORFs were compared with the already-known proteins by FASTA. As a result, ORF 1 and ORF 2 showed homologies to alanine racemase, and an ORF 3 showed no homology to other proteins. According to the number of the identical residues and the homology to alanine racemase, the ORF 1, which was composed of 1,230 bp, was cloned from the genome of *P. putida* IFO 12996. The ORF 1 from *P. putida* IFO 12996 was overexpressed in *E. coli* BL 21(DE3)/pLysS. After Ni^{2+} affinity chromatography, the protein was purified to near homogeneity in SDS-polyacrylamide gel electrophoresis. The deduced molecular mass of the protein was 45 kDa, and it was agreed with the molecular mass of BAR (42 kDa) including His×6 tag (3 kDa).

TABLE 3.1. Substrate specificity of BAR

Substrate	Relative activity (%)
L-Lys	100
L-Arg	65
L-Ala	33
L-Ser	20
L-Met	14
L-Cys	14
L-Leu	3.3
L-His	1.6
L-Phe	2.9×10^{-1}
L-Pro	1.3×10^{-1}
L-Thr	6.9×10^{-2}
L-Asn	5.4×10^{-2}
L-Asp	1.1×10^{-2}
L-Ile	6.6×10^{-3}
L-Trp	6.6×10^{-3}
L-Val	3.3×10^{-3}

Besides, as shown in TABLE 3.1, the protein from ORF 1 from *P. putida* IFO 12996 showed apparent racemase activity for lysine and arginine which were excellent substrates of BAR. Therefore, it was concluded that the ORF 1 from *P. putida* IFO 12996 encoded BAR. In addition, it was revealed that BAR showed slight racemase activity toward some amino acids including aromatic and acidic amino acids. The nucleotide sequence of BAR has already been submitted to DNA Data Bank of Japan (accession number; BD373122) as a part of a patent by other researchers (16); however, the slight racemase activity for some more amino acids was newly revealed in this study.

3.3.2. D-Amino acid dipeptide production in the coupling reaction of purified EcDdlB and BAR

As the cloned BAR showed broad substrate specificity, D-amino acid dipeptide production in the coupling reaction with EcDdlB and BAR was performed. In this reaction, D-amino acid was supplied from L-amino acid by BAR, subsequently, D-amino acid dipeptide was produced from D-amino acid by EcDdlB. Therefore, an inexpensive material *i.e.* L-amino acid could be used for D-amino acid dipeptide production.

In the coupling reaction, the synthetic yield of D-alanyl-D-alanine, D-serinyl-D-serine, D-cysteinyl-D-cysteine, and D-threonyl-D-threonine calculated from the theoretical molar amount of each dipeptides reached 44, 36, 9, and 1%, respectively (FIG. 3.1). Since there was no L-form dipeptide synthesis through the reaction, it was confirmed that EcDdlB was D-enantiomer specific, and chirality pure D-amino acid dipeptide was specifically produced in this reaction. Thus, it was confirmed that the

coupling reaction progressed as expected. Then, this coupling reaction was applied into the resting cell reaction.

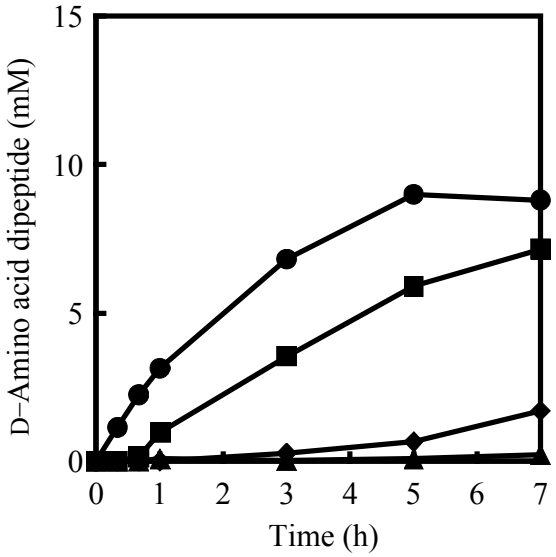

FIG. 3.1. Production of D-amino acid dipeptides in the coupling reaction. Reaction mixtures containing L-alanine, L-serine, L-threonine, and L-cysteine were incubated at 37ºC. The concentration of phosphate released was detected at each reaction. Symbols: circles, D-alanyl-D-alanine; squares, D-seryl-D-serine; triangles, D-threonyl-D-threonine; diamonds, D-cysteinyl-D-cysteine.

3.3.3. D-Amino acid dipeptide production in the resting cell reaction

Since the coupling reaction was performed successfully, it was applied into resting cell reaction system to utilize ATP regeneration ability of *E. coli* cell. In this system, ATP would be regenerated from glucose through glycolytic pathway; therefore the starting materials required for this system would be only L-amino acid and glucose.

As shown in FIG. 3.2, racemization of L-alanine and ligation of D-alanine without any addition of ATP were progressed, and D-alanyl-D-alanine (14.8 mM, 74% (mol/mol) yield) was successfully synthesized in this system. As for the reaction velocity of racemization and ligation, D-alanyl-D-alanine was gradually synthesized until the end of the reaction, following consumption of racemic D,L-alanine during the reaction time

from one hour to 27 h, therefore it was suggested that racemization progressed much faster than ligation.

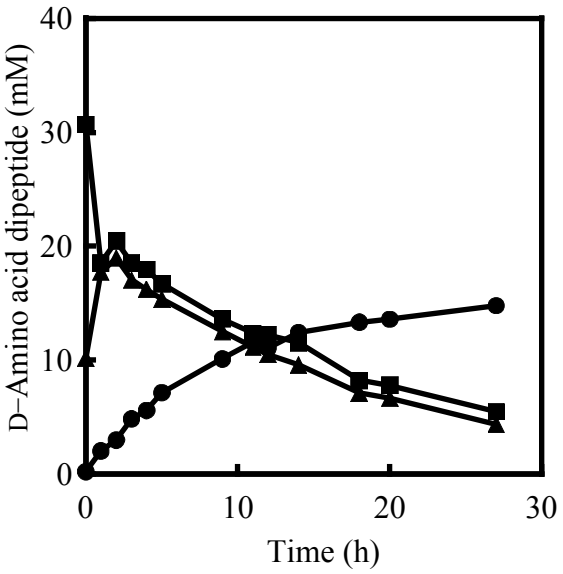

FIG. 3.2. Production of D-alanyl-D-alanine in the resting cell reaction. Symbols: circles, D-alanyl-D-alanine; squares, L-alanine; triangles, D-alanine.

3.4. Discussion

In this chapter, the gene encoding broad specificity amino acid racemase from *P.putida* IFO 12996 was cloned. BAR showed high homology to bacterial alanine racemases; therefore, BAR was considered as a kind of alanine racemase. Bacterial alanine racemase catalyzes the racemization of L-alanine to D,L-alanine, and subsequently, D-alanine is supplied to the biosynthesis of peptidoglycan. Therefore, BAR is considered to have the same function in *P. putida* IFO 12996. However, there is no report about the existence of D-amino acid except D-alanine or D-glutamate in the cell wall of *P. putida*. On the other hand, it has been reported that L-lysine was partially metabolized through D-lysine and suggested that there was lysine racemase in *P.*

putida (17). Therefore, BAR, which showed the highest activity toward lysine, would participate in the L-lysine metabolic pathway.

In the coupling reaction with purified EcDdlB and BAR, it was confirmed that D-amino acid dipeptide could be synthesized from L-amino acid as starting material. Since D-amino acids are still expensive materials, use of L-amino acid must contribute to cost effective production of D-amino acid dipeptide utilizing Ddl. Moreover, BAR is able to supply various D-amino acids from L-amino acids; therefore BAR would be utilized as D-amino acid supplying enzyme not only in D-amino acid dipeptide synthesis but also in synthesis of other D-amino acid derivatives. However, the synthetic yield of each D-amino acid dipeptide from L-amino acid was reduced to approximately 50% compared with that from D-amino acid. In the case of L-cysteine and L-allothreonine as substrate, it was supposed that supply of D-amino acid was the rate-limiting step because BAR showed weak activity to these substrates. On the other hand, racemization of L-alanine and L-serine progressed much faster than dipeptide synthesis (data not shown), therefore it was not explained by activity of BAR why the yields of D-alanyl-D-alanine and D-serinyl-D-serine were reduced in the coupling reaction. In this reaction, synthesized D-amino acid would be a substrate not only for EcDdlB but also for BAR, therefore it was supposed that D-amino acid was mainly used as a substrate for BAR, and EcDdlB would be able to utilize less amount of D-amino acid resulting low synthetic yield. Taken together, it was suggested that activity balance of EcDdlB and BAR was important factor for the yield of D-amino acid dipeptide.

Although D-amino acid was successfully supplied from L-amino acid by BAR, requirement of ATP was still major problem in production process with EcDdlB. Then glycolytic pathway that produces ATP from glucose was focused. In order to utilize cell glycolytic pathway, fermentative process or resting cell reaction were needed.

Since L-amino acid and glucose were supposed to be carbon and nitrogen source for growth of *E. coli*, resting cell reaction was considered as more suitable than fermentative process. As expected, L-amino acid was mainly utilized for D-amino acid dipeptide synthesis, and the synthetic yield of D-alanyl-D-alanine reached 74% (mol/mol) that was equal to that in the reaction using purified EcDdlB with sufficient amount of D-alanine and ATP. In this resting cell reaction, ATP was only supplied through glycolytic pathway utilizing glucose as substrate, therefore it was confirmed that ATP regeneration of *E. coli* cell was capable to supply an efficient amount of ATP to the reaction of EcDdlB. Utilizing this resting cell reaction, the major problem, such as requirement of expensive D-amino acid and ATP, was solved, and construction of effective production process for D-amino acid dipeptide was achieved.

3.5. References

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Chapter 4

Random Mutagenesis on Broad Specificity Amino Acid Racemase from *Pseudomonas putida* IFO 12996 for Tryptophan Racemization

4.1. Introduction

D-Amino acids have been considered as biologically inactive substances, although L-amino acids are components of proteins and play important roles in the metabolism. However, almost all the bacteria synthesize and utilize D-amino acids, such as D-alanine, D-glutamate, D-valine, and D-phenylalanine, as the components of their cell membrane (1, 2) or antibiotics (3-5). Moreover, D-aspartate was discovered in mammalian cells, and it is suggested that the formation of D-aspartate concerned to aging (6-9). Therefore, D-amino acids are no longer biologically inactive but are important substances with diverse bioactivity. Besides, the enzymes acting on D-amino acids are recently focused.

Among those enzymes, broad specificity amino acid racemase from *Pseudomonas putida* IFO 12996 (BAR) is a unique enzyme because of its broad substrate specificity for various amino acids. In previous study in chapter 3, the gene encoding BAR was cloned and overexpressed in *E. coli*. The recombinant BAR was capable to recemize almost all the standard amino acids, and effectively supplied D-alanine, D-serine, D-cystein, D-threonine from the L-form of each amino acid to the reaction of Ddl to produce D-amino acid dipeptides. Therefore, it is considered that BAR is useful enzyme to supply D-amino acid to various production processes of

D-amino acid related compounds. However, the activity of BAR for aromatic and acidic amino acid was extremely low. Especially, amino acid racemase acting on aromatic amino acid was not reported, but it was expected that aromatic amino acid racemization activity might be readily appended to BAR by mutagenesis because of its broad substrate specificity. Then, random mutagenesis was performed on *bar* to obtain more useful BAR derivatives that show high activity for aromatic amino acid, especially for tryptophan in this study.

4.2. Materials and methods

4.2.1. Bacterial strains, plasmids, and culture conditions

P. putida IFO 12996 and *Escherichia coli* BL21 (DE3)/ pLysS were purchased from the Institute of Fermentation Osaka (Osaka, Japan) and Merck KGaA (Darmstadt, Germany), respectively. *E. coli* JM 109 was supplied from Nippon Gene (Tokyo, Japan), and tryptophan auxotroph *E. coli* JM 101: Δ*tna*A, Δ*trp*ABCDE was the thankful gift from Bio Frontier Laboratories, Kyowa Hakko Kogyo (Tokyo, Japan). Plasmid vector pET 21a (+) and pSTV 28 were from Merck KGaA and Takara Bio (Mie, Japan).

E. coli strains were grown in LB medium at 37 °C, with 120 rpm rotary shaking for 16 h. In the case of *bar* expression, recombinant *E. coli* BL21 (DE3)/pLysS harboring *bar* was grown in LB medium containing 50 μg/ml ampicillin, 25 μg/ml chloramphenicol, and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30 °C, with 120 rpm rotary shaking for 16 h.

4.2.2. Chemicals and materials

D-Amino acids were purchased from Kanto Kagaku (Tokyo, Japan) or Kokusan

Kagaku (Tokyo, Japan). 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide and 4-amino antipyrine were from Sigma-Aldrich (St. Louis, MO, USA). His-Trap HP 1-ml column, PD-10 column, and ÄKTA explorer 10S were from Amersham Bioscience (NJ, USA). Model 550 microplate reader was purchased from Bio-Rad Laboratories (CA, USA). L-7000 series high-performance liquid chromatography (HPLC)-equipped WH-C18A (3 μm) column was from Hitachi High-Technologies (Tokyo, Japan). All other chemicals were of reagent grade and obtained from commercial sources.

4.2.3. Gene cloning

The gene encoding BAR was amplified by polymerase chain reaction using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) with *Nde*I and *Eco*RI restriction sites at upstream and downstream of bar, respectively. After the digestion by *Nde*I and *Eco*RI, amplified *bar* was ligated to pET 21a (+) vector using DNA Ligation kit version 2.1 (Takara Bio) and introduced into *E. coli* JM 109 for plasmid isolation. The isolated recombinant plasmid harboring *bar* was introduced into *E. coli* BL 21(DE3)/pLysS.

4.2.4. Purification procedure of His×6-tagged BAR

The recombinant BAR with His×6 tag was produced in recombinant *E. coli* BL 21(DE3)/pLysS cells. The cells were harvested at 3,000×*g* for 10 min, and the cell pellet was treated with 5 ml of BugBuster Protein Extraction Reagent (Merck) for 20 min, and then centrifuged at 16,000×*g* for 30 min, and the supernatant was collected as cell-free extract.

The cell-free extract was applied onto a His-Trap HP 1-ml column equilibrated with 100 mM Tris-HCl (pH 8), 150 mM NaCl, and 10 mM imidazole, and the column was washed with 10 column volumes of the same buffer. The His×6-tagged BAR was

eluted by linear gradient with 100 mM Tris-HCl (pH 8), 150 mM NaCl, and 500 mM imidazole and desalted on a PD-10 column equilibrated with 100 mM Tris-HCl (pH 8.5).

4.2.5. Random mutagenesis on bar and construction of the mutant library

Ramdom mutagenesis on bar was carried out by error-prone PCR method. The PCR reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.2 mM each of dNTPs, 0.5 pmol/μl each of primers, 800 pg/μl template DNA, and 25 mU/μl Taq DNA polymerase (Takara Bio). The amplified PCR product with *Eco*RI and *Bam*HI restriction sites at 5' or 3'-terminus were restricted and ligated to pSTV 28 vector. Then, *E. coli* JM 101: Δ*tna*A, Δ*trp*ABCDE was transformed with resulting plasmids.

4.2.6. Screening of mutant BAR with tryptophan racemase activity

First, *E. coli* JM101: ΔtnaA, ΔTrpABCDE harboring mutated *bar* were grown in M9 medium containing 5 mg/l D-tryptophan and 50 mg/l casamino acids at 37 °C for 16 h, and then spread onto M9 agar plate containing the same additives and incubated at room temperature until colonies were formed. The colony-forming mutants were applied to the second screening.

In the second screening, the mutants were inoculated to 800 μl of LB medium containing 25 μg/ml chloramphenicol and 0.1 mM IPTG and incubated at 37 °C for 16 h. The grown cells were harvested at 2,800×*g* for 10 min, washed twice with 100 mM Tris-HCl (pH 8.5), and resuspended to the same buffer. Portions (50 μl) of the cell suspensions were mixed with 50 μl of 30 mM L-tryptophan solution and incubated at 37 °C for 16 h, then 100 μl of colorimetric assay mixture consisted of 10 mM Tris-HCl

(pH 8.5), 1 U / ml D-amino acid oxidase, 100 U / ml peroxidase, 5 mM phenol and 1 mM 4-amino antipirine was added, and the absorbance at 490 nm was measured. The selected mutant *bar*s from the second screening were subcloned into *E. coli* BL 21(DE3)/pLysS as mentioned above.

4.2.7. Site-directed mutagenesis

The full length of the recombinant plasmid harboring wild-type *bar* was amplified by PCR using KOD-plus DNA polymerase with a pair of mutagenic primers to introduce the site-directed mutagenesis. In this PCR amplification, each primer contained 20 bp of overlapping region and 10 bp of each annealing region, and one of them had mutation between overlapping region and annealing region. The amplified PCR product was directly introduced into the *E. coli* JM 109, and the linear PCR product was cyclized in the host cell. After the plasmid isolation, occurrence of mutation was confirmed by DNA sequencing.

4.2.8. Tertiary structure prediction

The amino acid sequence of wild-type or mutant BAR was submitted to ESyPred3D (10) (http://www.fundp.ac.be/urbm/ bioinfo/esypred/) to predict tertiary structure. The predicted tertiary structures were visualized using VMD molecular visualization program (11).

4.3. Results

4.3.1. Introduction of random mutagenesis on *bar*

The mutant library of BAR was constructed by the method of error-prone PCR.

Although the mutation ratio is generally used to evaluate the mutant library by checking DNA sequence, it is not suitable for evaluation of a number of mutants. Then, a shift of the L-leucine racemizing activity was measured to confirm introduction of random mutagenesis on *bar*. Although L-leucine was a poor substrate of BAR, racemizing rate of L-leucine was more suitable rather than that of good substrates for quantitative analysis of a number of mutants. As shown in FIG. 4.1, almost all the arbitrarily selected mutants showed shifted racemase activity compared with wild-type BAR; therefore, it was confirmed that mutations were certainly introduced to *bar*.

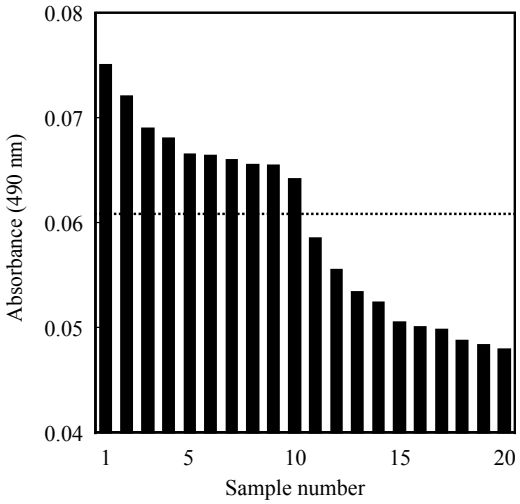

FIG. 4.1. Inactivation ratio of randomly mutated BARs. 20 mutants were selected from mutant library of BAR generated by random mutagenesis, and applied to colorimetric assay. The absorbance of wild-type BAR on colorimetric assay was shown in dotted line.

4.3.2. Screening of mutant BAR with high tryptophan racemase activity

In the first screening of mutant BAR, the complementation of L-tryptophan auxotrophy was employed as indication for the increased tryptophan racemase activity. On the M9 agar plate containing D-tryptophan, only the L-tryptophan auxotroph *E. coli* JM 101: Δ*tna*A, Δ*trp*ABCDE harboring mutant *bar* with increased tryptophan racemase

activity was able to convert sufficient amount of D-tryptophan to L-tryptophan which was required for their growth. Through this screening, 80 positive mutants were isolated from 30,000 mutants.

The isolated 80 positive mutants were applied onto the second screening mentioned in the "Materials and methods". As shown in FIG. 4.2, there was a positive mutant with the highest increase of 210% activity. However, only one mutant was not enough to investigate the diversity of positive mutations; then five positive mutants whose tryptophan racemase activities markedly increased was isolated, and the mutants were named mutants 1, 2, 3, 4, and 5, respectively.

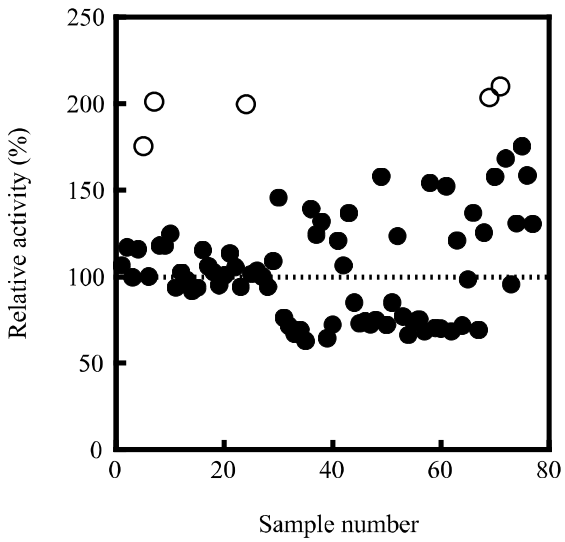

FIG. 4.2. Colorimetric assay of positive mutants from the first screening.
The relative activities indicate the absorbance of 80 positive mutants when that of wild-type BAR was regarded as 100%.

4.3.3. Amino acid substitutions and specific activities of mutant BARs

The specific activities of the mutants for tryptophan, phenylalanine, lysine, and alanine were measured, and the amino acid substitutions were determined by DNA sequencing (TABLE 4.1). The mutants showed five- to 20-fold increased specific

activities for tryptophan; besides, the specific activities of mutant 4 were also increased when the other amino acids were employed as substrates. On the other hand, the specific activity for tryptophan was selectively increased in mutants 1, 2, 3, and 5. Therefore, it was suggested that there were two types of mutants. From the analysis of the amino acid substitutions, mutant 4 which only showed the increased specific activity for overall amino acids had I384M extra substitution, although all the mutants had the amino acid substitution at Y396.

TABLE 4.1 Amino acid substitutions and specific activities of wild type and five mutants

Amino acid substitution	Specific activity (nmol/min/mg)[a]							
	Trp		Phe		Lys		Ala	
none (wild-type)	5.88	(1.0)	8.38	(1.0)	132000	(1.0)	7820	(1.0)
Y293S, Y301S, **Y396C**	26.7	(4.5)	24.9	(3.0)	458000	(3.5)	14100	(1.8)
I83L, D361V, **Y396C**	30.3	(5.2)	25.7	(3.1)	473000	(3.6)	12100	(1.5)
L126H, **Y396C**	52.4	(8.9)	43.6	(5.2)	477000	(3.6)	14400	(1.8)
I384M, **Y396C**	115	(20.0)	79.1	(9.4)	761000	(5.8)	18200	(2.3)
Y396H	52.8	(9.0)	4.28	(0.5)	16800	(0.1)	3400	(0.4)

[a] Values in parentheses indicate the ratio of specific activity compared with that of wild type Bar.

Then, site-directed mutagenesis was performed at Y396 and I384. The generated Y396C, Y396H, and I384M single mutants showed the same tendencies in increase of activity (TABLE 4.2). Therefore, it was definitely confirmed that Y396C and Y396H substitutions concerned to the selective increase of specific activity for tryptophan, and I384M substitution concerned to the increase of specific activities for overall amino acids.

TABLE 4.2 Specific activities of wild type and three mutants generated by site-direct mutagenesis

Amino acid substitution	Specific activity (nmol/min/mg)[a]			
	Trp	Phe	Lys	Ala
wild-type	5.88 (1.0)	8.38 (1.0)	132000 (1.0)	7820 (1.0)
Y396C	48.7 (8.3)	7.06 (0.8)	32150 (0.2)	825 (0.1)
Y396H	13.6 (2.3)	14.5 (1.7)	74500 (0.6)	1700 (0.2)
I384M	124 (21.0)	74.4 (8.9)	223000 (1.7)	26000 (3.3)

[a] Values in parentheses indicate the ratio of specific activity compared with that of wild type Bar.

4.3.4. Racemization of L-tryptophan using modified BAR

The modified BARs with the high tryptophan racemizing activity were obtained by Y396 and I384 substitution, and then racemization of L-tryptophan was tested. As shown in Fig. 4.3, Y396C and wild-type BAR partially racemized L-tryptophan, and the proportions of D-tryptophan in the reaction mixture were 12.5 and 6%, respectively. On the other hand, I384M mutant BAR racemized almost all L-tryptophan to D,L-tryptophan in the 24-h reaction.

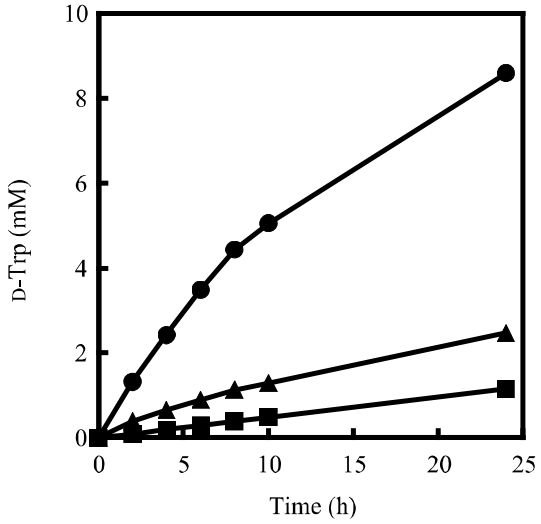

FIG. 4.3. L-Trp racemization with modified BAR. The modified and wild-type BARs were incubated with 20 mM L-Trp. After the incubation, racemized D-Trp was measured by HPLC. Symbols indicate the amount of produced D-Trp with I384M mutant; circle, Y396C mutant; triangle, and wild-type BAR; square.

4.4. Discussion

In this chapter, it was suggested that the amino acid substitutions at Y396 and I384 increased the tryptophan racemizing activity of BAR, but the increased activities were achieved in the different mechanism. In the comparison with I384M single mutant and the double (I384M, Y396C) mutant obtained from random mutagenesis, I384M single mutant showed the higher specific activity for tryptophan, and the apparent effect of Y396C substitution was not observed on the double mutant. This suggests that the effect of the amino acid substitution at I384 on the activity of BAR is much higher than that of the amino acid substitution at Y396. However, Y396 is the only residue concerning to the substrate specificity of BAR; therefore, the further amino acid substitutions at Y396 are expected to contribute to elucidate the substrate recognition mechanism of BAR.

Among bacterial alanine racemases, the tertiary structures of alanine racemase from *Bacillus stearothermophilus* (12) and *Pseudomonas aeruginosa* (13) were reported so far (Fig. 4.4A, B). Both reported tertiary structures showed that the monomer of alanine racemase was consisted of two domains, and the N-terminal domain mainly constructed the active center. However, it was reported about alanine racemase from *B. stearothermophilus* that Y354, which is located in the region with tandem two α-helixes in C-terminal domain, was the residue to concern to construction of the active center, and the residue was considered to immobilize PLP cofactor. Because the Tyr residues were conserved in both alanine racemases from *B. stearothermophilus* and *P. aeruginosa* (Y341), it was suggested that other alanine racemases would have the same Tyr residue. In the C-terminal sequence of BAR, Y396 was the only Tyr residue, and it was supposed to be located in the same region as Tyr residues in other alanine

racemases from the predicted tertiary structure (Fig. 4.4C). Then, it is considered that Y396 partially has interaction with PLP, and the substitution of Y396C or Y396H would change the conformation of active center to be more suitable for tryptophan. Besides, I384 was located at the same tandem two α-helixes; therefore, it is supposed that I384M substitution influences the formation of active center through the translocation of Y396 or the tandem two α-helixes themselves.

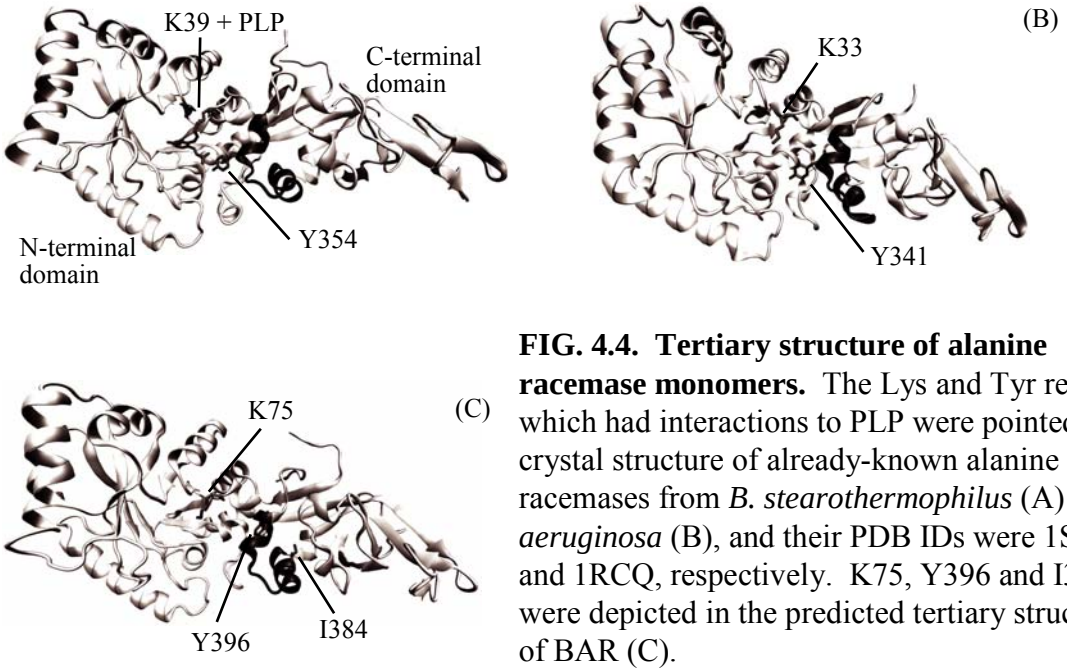

FIG. 4.4. Tertiary structure of alanine racemase monomers. The Lys and Tyr residues which had interactions to PLP were pointed in the crystal structure of already-known alanine racemases from *B. stearothermophilus* (A) and *P. aeruginosa* (B), and their PDB IDs were 1SFT and 1RCQ, respectively. K75, Y396 and I384 were depicted in the predicted tertiary structure of BAR (C).

In the study of the L-tryptophan racemization, 20 mM L-tryptophan was partially racemized, and 2.5 mM D-tryptophan was formed by Y396C mutant BAR. On the other hand, almost all the initial L-tryptophan was racemized, and 8.6 mM D-tryptophan was formed by I384M mutant in 24 h. Although some slight racemization activities on tryptophan were reported, it was a novel reports that I384M mutant BAR showed high racemizing activity of tryptophan as an enzyme. Moreover, it is expected that I384M mutant BAR could effectively supply not only D-tryptophan but also other aromatic, neutral, and basic D-amino acids to various production process of D-amino acid related compounds including D-amino acid dipeptides.

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Chapter 5

Substrate Specificity of Thermostable D-Alanine-D-alanine Ligase from *Thermotoga maritima* ATCC 43589

5.1. Introduction

Bacterial peptidoglycan is an important biopolymer that protects the bacterial cell from environmental stress. The basic unit of peptidoglycan consists of N-acetyl glucosamine, N-acetyl muramic acid, and pentapeptide (L-Ala-D-Glu-DAP or L-Lys-D-Ala-D-Ala), and the peptide bond between D-alanine and D-alanine is cleaved to construct the cross-linking in the peptidoglycan. In the biosynthesis of peptidoglycan, synthesis of D-alanyl-D-alanine dipeptide is catalyzed by D-alanine-D-alanine ligase (Ddl) (1-3), and subsequently D-alanyl-D-alanine is ligated to UDP-N-acetylmuramoyl tripeptide.

Among bacterial Ddls, there are three types of mutant Ddls, named VanA, VanB, and VanC respectively. They have been isolated from vancomycin-resistant enterococci, and the mutants constitute an identical evolutionary group on the phylogenetic tree. These mutants are different from other Ddls as to substrate specificity. VanA and VanB synthesize D-alanyl-D-lactate and VanC synthesizes D-alanyl-D-serine (4-6). Therefore, it is thought that the altered substrate specificities are inherent characteristics of VanA, VanB, and VanC, and that Ddl recognize solely D-alanine as substrate.

However, in othe previous study on the synthesis of D-amino acid dipeptides, the

author obtained a novel Ddl with broad substrate specificity from *Thermotoga maritima* ATCC43589 (TmDdl) (Chapter 2) (7). Although TmDdl is thought not to belong to the group of VanA, VanB, or VanC on the phylogenetic tree (Fig. 5.1), TmDdl employed D-serine, D-threonine, D-cysteine, and glycine in addition to D-alanine as substrate in the reaction at 37°C. Ddl, which is not a VanA, VanB, or VanC type mutant, recognized not only D-alanine but also other D-amino acids. Then the substrate specificity at near the optimal temperature for the growth of *T. maritma* ATCC 43589 was investigated for further characterization of the enzyme function.

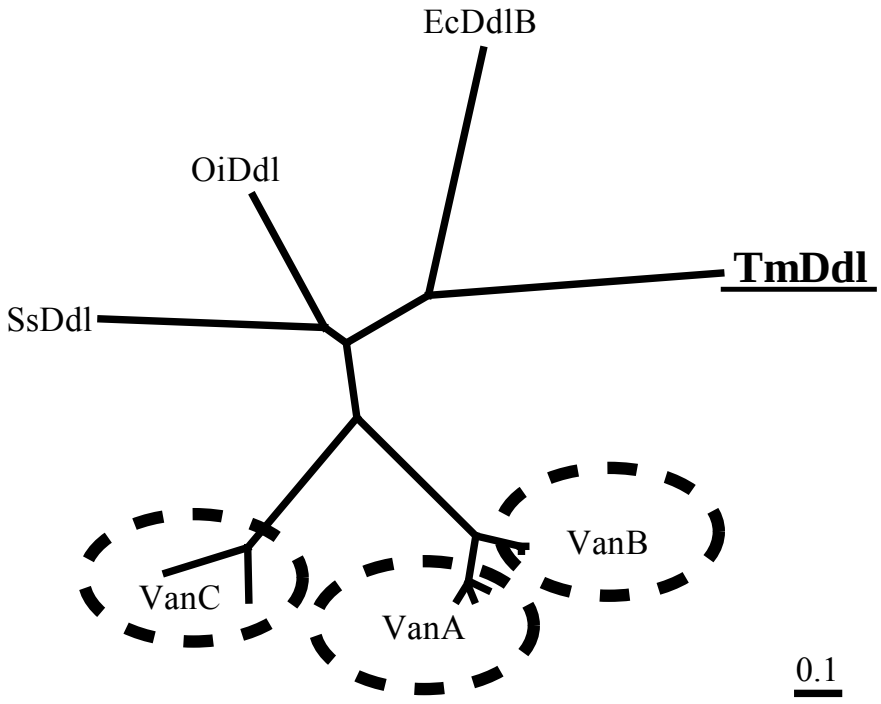

Fig. 5.1. Phylogenetic Tree of Ddl, VanA, VanB, and VanC. The amino acid sequences of four Ddls, four VanAs, four VanBs, and two VanCs were submitted to the sequence alignment program Clustal W. EcDdlB, OiDdl, SsDdl, indicate Ddl from *E. coli* K 12, *Oceanobacillus iheyensis* HTE 831, and *Synschocystis* sp. PCC 6803 respectively.

5.2. Materials and methods

5.2.1. Bacterial strains and culture conditions

Genomic DNA of *T. maritima* ATCC 43589 was purchased from American Type Culture Collection (Manassas, VA, USA). *Escherichia coli* BL21 (DE3) and JM 109 were purchased from Merck KGaA (Darmstadt, Germany) and Nippon gene (Tokyo, Japan), respectively. *E. coli* strains were grown in LB medium at 37°C, with 120 rpm rotary shaking for 16 h. In the case of gene expression, recombinant *E. coli*s BL21 (DE3) harboring *Tmddl* was grown in LB medium containing 25 μg / ml kanamycin at 30°C with 120 rpm rotary shaking for 16 h. Expression of the gene was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG)

5.2.2. Chemicals and materials

D-Amino acids were purchased from Sigma (St. Louis, MO, USA). 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) was from Merck KGaA. His-Trap HP 1-ml column, PD-10 column and ÄKTA explorer 10S were from Amersham bioscience (N. J., USA). L-7000 series high performance liquid chromatography was from Hitachi high-technologies (Tokyo, Japan). WH-C18A column (Hitachi high-technologies) was used for HPLC analysis. All other chemicals were reagent grade and obtained from commercial sources.

5.2.3. Gene cloning

The gene encoding TmDdl was amplified by polymerase-chain-reaction using KOD plus DNA polymerase (Toyobo, Osaka, Japan) with LIC adopter sequence. The amplified DNA fragment with LIC adopter sequence was introduced pET 30 Xa / LIC

vector (Merck KGaA) according to the instructions provided by manufacturer. This recombinant plasmid was named pTmDdl and used to produce N-teminal His×6 tagged TmDdl. The recombinant plasmid was introduced into *E. coli* JM 109, and the sequence of the gene was confirmed after plasmid isolation. Subsequently, the sequenced plasmid was introduced into *E. coli* BL21(DE3) for gene expression.

5.2.4. Overexpression of *tmddl* and enzyme purification

E. coli BL21(DE3) / pTmDdl was harvested at 3000 × *g* for 10 minutes and the cell pellet was treated with 5ml of BugBuster Protein Extraction Reagent (Merck KGaA) for 20 minutes, then centrifuged at 16,000×*g* for 30 minutes and the supernatant was collected as cell-free extract. The cell-free extract was applied onto a HisTrap HP 1-ml column equilibrated with 50 mM Tris-HCl (pH 8.0), 500 mM NaCl and 10 mM imidazole, and the column was washed with 10 column volumes of the same buffer. The His×6 tagged protein was eluted by linear gradient with 50 mM Tris-HCl (pH 8.0), 500 mM NaCl and 500 mM imidazole and desalted on a PD-10 column equilibrated with 50 mM Tris-HCl (pH 8.0). The desalted His×6 tagged protein was applied onto a HiTrap Q HP 1-ml column equibrated with 50 mM Tris-HCl (pH 8.0), and the column was washed with 10 column volumes of the same buffer. The His×6 tagged protein was eluted by linear gradient with 50 mM Tris-HCl (pH 8.0), 500 mM NaCl and desalted on a PD-10 column equilibrated with 50 mM Tris-HCl (pH 8.0) again.

5.2.5. Enzyme assay

The reaction mixture for TmDdl activity assay was consisted of 100 μg /ml of the purified TmDdl, 10 mM D-alanine, 5 mM ATP, 10 mM $MgSO_4$, and 50 mM Tris-HCl (pH 8.0). The specific activity was determined by released phosphate

colorimetry using Determinar L IP (Kyowa Medex, Tokyo) by measuring the absorbance at 595 nm. In addition, the synthesized D-alanyl-D-alanine was quantified by HPLC.

5.2.6. HPLC analysis

A WH-C18A (3 μm) column (Hitachi High Technologies, Tokyo) was used for quantification of D-alanyl-D-alanine by HPLC. A gradient elution of solvent A [50 mM KH_2PO_4 (pH 2.7 by phosphate):acetonitrile:methanol=18:1:1] and solvent B [50 mM KH_2PO_4 (pH 2.7 by phosphate):acetonitrile:methanol=12:7:1] was used with the following program: 0 to 24 min; a linear increase in solvent B to A:B of 55:45, held to 30 min, 30 to 50 min; a linear increase to A:B of 0:100, held to 55 min. After each gradient elution, the column was washed with solvent C [water: tetrahydrofuran; acetonitrile=1:1:3] for 2 min and re-equibrated with solvent A for 20 min. The flow rate was maintained at 0.5 ml / min, and the column temperature was kept at 40°C. After separation, UV absorption was monitored at 340 nm. D-Alanyl-D-alanine was derivetized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide and subjected to HPLC.

5.3. Results

5.3.1. Thermal and pH stability of TmDdl

TmDdl was expressed as a His×6 tag fusion protein, and purified to homogeneity by Ni^{2+} affinity chromatography.

The optimal temperature for the growth of *T. maritima* ATCC 43589 is 80ºC; therefore, it was expected that TmDdl would show high thermal stability. Thus, the thermal stability of His×6-tagged TmDdl purified from the recombinant *E. coli* was

tested. In addition, His×6-tagged Ddl from *E. coli* K 12 (EcDdlB) was also tested as a negative control.

As shown in Fig. 5.2, TmDdl was highly thermostable and there was no decrease in activity between 10ºC and 90ºC. On the other hand, EcDdlB lost its activity over 60ºC. Although TmDdl showed less activity than EcDdlB at 37ºC, the activity of TmDdl increased with the rise in temperature. At 80ºC, the optimal temperature for growth of *T. maritima* ATCC 43589, TmDdl showed almost equal activity to that of EcDdlB at 37ºC.

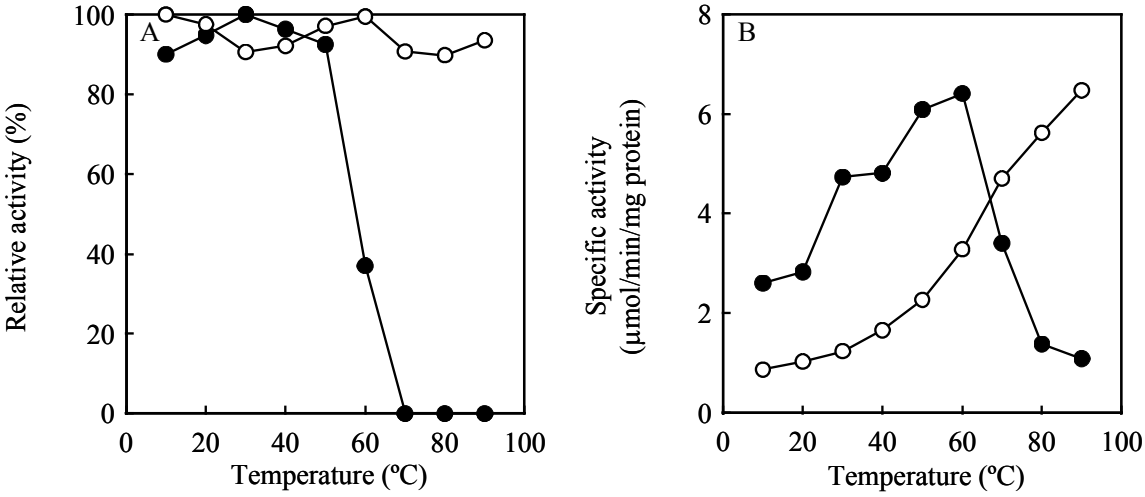

Fig. 5.2. Thermal Stability of TmDdl. The reaction mixture was incubated at 37ºC for 20 min. Before the reaction, purified Ddl was incubated at several temperatures for 15 min (A). The reaction was carried out at several temperatures for 20 min (B). The open and closed circles indicate the activity of TmDdl and EcDdlB, respectively.

5.3.2. Substrate specificity of TmDdl

Despite the thermal stability of TmDdl, the ATP required for the reaction was easily degraded at over 80ºC. Therefore, the substrate specificity of TmDdl was investigated at 60ºC. It was evaluated by measuring the amount of released inorganic

phosphate produced at the equal molar amount of synthesized D-alanyl-D-alanine in the reaction of TmDdl. As shown in Table 5.1, it was found that TmDdl employed 15 kinds of D-amino acids, such as D-alanine, D-cysteine, D-serine, D-threonine, D-lysine, D-glutamine, D-histidine, D-arginine, D-valine, D-isoleucine, D-methionine, D-leucine, D-phenylalanine, D-proline, and D-asparagine, as substrate at the high temperature. Moreover, TmDdl recognized glycine and β–alanine, which were not chiral amino acids, although L-amino acid was not employed as substrate (data not shown). In the reaction at 37ºC, TmDdl showed activity only for D-alanine, D-serine, D-threonine, D-cysteine, and glycine. Therefore it was found that the substrate specificity was expanded at the high temperature, near the optimal growth temperature of *T. maritima* ATCC 43589.

5.4. Discussion

In this chapter, it was found that the substrate specificity of TmDdl was expanded at the high temperature, near the optimal growth temperature of *T. maritima* ATCC 43589, while TmDdl showed activity only for D-alanine, D-serine, D-threonine, D-cysteine, and glycine at 37ºC. The CD spectrum of TmDdl at 60ºC slightly differed from that at 37ºC (data not shown), therefore it is thought that the preferable tertiary structure and the high temperature itself enhanced the reaction velocity of TmDdl. The broad substrate specificity of TmDdl at 60ºC was detected as a result of enhancement of the reaction velocity.

In comparison with other Ddls, the maximum specific activity of TmDdl for D-alanine (5.5 μmol/min/mg) was slightly lower than that of Ddl from *E. coli* (31 or 12.3 μmol/min/mg) (8) and *Salmonella typhimurium* (16.4 μmol/min/mg) (9), and the specific activities of TmDdl for other D-amino acids were low further. Therefore, it is

thought that D-amino acid dipeptides, except for D-alanyl-D-alanine, are not synthesized in the living cell of *T. maritima* because of the low specific activity of TmDdl for the other D-amino acids and few supplementations of D-amino acids in the living cell. Of course, it is supposed that Ddls synthesize only D-alanyl-D-alanine in the bacterial cells, but as for the individual function of the enzyme, there should be some Ddls which show ligase activities for various D-amino acids.

TABLE 5.1 Specific Activity of TmDdl

Substrate	Specific activity (nmol/min/mg protein)	
	60ºC	37ºC
D-Alanine	5.5×10^3 (100)[a]	4.2×10^2 (7.5)
D-Serine	8.8×10^2 (16)	7.4×10^2 (13)
Glycine	2.0×10^2 (3.6)	4.3×10^2 (7.8)
D-Threonine	1.2×10^2 (2.2)	1.2×10^2 (2.2)
D-Cysteine	1.6×10^3 (29)	7.4×10^2 (13)
D-Tyrosine	N.D.[b]	N.D.
D-Asparagine	12 (0.22)	N.D.
D-Glutamine	31 (0.56)	N.D.
D-Aspartate	3 (0.051)	N.D.
D-Glutamate	1 (0.012)	N.D.
D-Lysine	33 (0.60)	N.D.
D-Arginine	24 (0.43)	N.D.
D-Histidine	27 (0.49)	N.D.
D-Valine	24 (0.43)	N.D.
D-Leucine	17 (0.31)	N.D.
D-Isoleucine	20 (0.36)	N.D.
D-Proline	16 (0.29)	N.D.
D-Phenylalanine	17 (0.31)	N.D.
D-Methionine	19 (0.34)	N.D.
D-Tryptophan	6 (0.10)	N.D.
β-Alanine	71 (1.3)	N.D.

[a] Values in parentheses indicate the ratio of specific activity compared with that for D-alanine.
[b] N.D., not detected.

Peptide synthesizing enzymes which belong to the ATP-grasp superfamily are focused on as biocatalysts for effective bioproduction of some peptides. Recently, Tabata *et al.* reported a novel L-amino acid ligase and constructed an effective bioprocess for producing L-alanyl-L-glutamine as a useful material for infusion (10). Therefore, it is expected that Ddls with broad substrate specificities from non-pathogenic bacteria, such as TmDdl, are widely utilized for bioproduction of D-form peptides.

5.5. References

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Chapter 6

D-Amino Acid Dipeptide Synthesis with a Thermostable ATP Regeneration System using Polyphosphate Kinase from *Thermosynechococcus elongatus* BP-1

6.1. Introduction

An ATP regeneration system is an important technique for industrial production processes utilizing ATP-dependent enzymes. Several ATP regeneration systems have been established using various enzymes, such as acetate kinase (1-5), polyphosphate kinase (6), or polyphosphate AMP phosphotransferase conjugated with adenylate kinase (7) or polyphosphate kinase (8). ATP regeneration systems are divided in two types depending on the source of adenosine scaffold. One type utilizes acetate kinase or polyphosphate kinase alone and regenerates ATP from ADP, and the other type regenerates from AMP via ADP. Therefore, a suitable ATP regeneration system is to be selected on the basis of the reaction mechanism of the enzyme coupled with it. By comparing with an ATP regeneration system utilizing two enzyme conjugations, the systems using acetate kinase or polyphosphate kinase are considered preferable because of their simplicity if only ATP regeneration from ADP is required. In addition, polyphosphate that requires polyphosphate kinase as a phosphate donor is easily available and an inexpensive material. Thereby, an ATP regeneration system utilizing polyphosphate kinase is considered as one of the most important systems for the industrial use of ATP-dependent enzymes.

Among ATP-dependent enzymes, glutathione synthetase (9), glutamine synthetase (10), L-amino acid α–ligase (11) and D-alanine-D-alanine ligase (12) are regarded as useful biocatalysts for producing D- or L-amino acid-related compounds, such as glutathione, theanine and D,L-dipeptide. In particular on the production of glutathione and theanine, ATP is effectively supplied from glucose through the glycolytic pathway (13-15). On the other hand, an enzymatic ATP regeneration system using polyphosphate kinase is also applied to the production processes utilizing these enzymes, and L-dipeptides have been effectively produced by the coupling reaction of L-amino acid α–ligase and polyphosphate kinase (16). These enzymes commonly consume ATP and subsequently release ADP and inorganic phosphate. Therefore, polyphosphate kinase that synthesizes ATP from ADP and polyphosphate is suitable for an ATP regeneration system for conjugating the reactions of these enzymes.

In the studies for the development of peptide function, D-amino acid peptides are expected to increase the functional variety and applications of peptides (17-21), and we have obtained a unique D-alanine-D-alanine ligase from a thermophile, *Thermotoga matirima* ATCC 43589 (TmDdl), for the production of various D-amino acid dipeptides previously (Chapter 5) (22). TmDdl showed a high thermostability and exerted a broad substrate specificity for various D-amino acids in a reaction at a high temperature. TmDdl had the broadest substrate specificity among the Ddls characterized thus far (12, 22), and it was expected that various D-amino acid dipeptides could be synthesized using TmDdl. However, this enzyme also required ATP for its reaction and released ADP and inorganic phosphate. Thus, it was considered that an ATP regeneration system using polyphosphate kinase is suitable for conjugating the reactions of TmDdl. However, the ATP regeneration systems reported thus far consisted of enzymes from mesophiles, and it was considered that it is difficult to operate the systems at high

temperatures.

Thus, we planned to establish a thermostable ATP regeneration system and focused on polyphosphate kinase from *Thermosynechococcus elongatus* BP-1 (TePpk) as a putative thermostable enzyme. In this study, the themostable ATP regeneration system was demonstrated using TePpk, and an effective method for the synthesis of D-amino acid dipeptide was examined with the system.

6.2. Materials and methods

6.2.1. Bacterial strains, genomic DNAs, plasmids and culture conditions

The genomic DNA of *T. elongatus* BP-1 was provided by Kazusa DNA Research Institute, and that of *T. maritima* ATCC 43589 was purchased from American Type Culture Collection (Manassas, VA, USA). *Escherichia coli* BL21(DE3) and JM 109 were purchased from Merck KGaA and Nippon Gene (Tokyo), respectively. Plasmid vector pET 21a (+) and 30 Xa / LIC were obtained from Merck. *E. coli* strains were grown in LB medium at 37°C with 120 rpm rotary shaking for 16 h. In the case of gene expression, recombinant *E. coli* BL21(DE3) harboring *Teppk*, *Tmddl* or both was grown in LB medium containing 50 μg/ml ampicilline or 25 μg/ml kanamycin at 30°C with 120 rpm rotary shaking for 16 h. The expression of each gene was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

6.2.2. Chemicals and materials

Sodium triphosphate penta-basic ($PolyP_3$) and sodium hexametaphosphate ($PolyP_{12-13}$) were purchased from Sigma (St. Louis, MO, USA). Hexasodium tetraphosphate ($PolyP_4$) was obtained from Junsei Kagaku (Tokyo, Japan). D-Alanine was purchased

from Kanto Kagaku (Tokyo). 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) was purchased from Merck KGaA (Darmstadt, Germany). A His-Trap HP 1-ml column, a PD-10 column and ÄKTA explorer 10S were obtained from GE Healthcare (Fairfield, CT, USA). An L-7000 series high-performance liquid chromatography system was obtained from Hitachi High-technologies (Tokyo). A WH-C18A column (Hitachi High-technologies) or Inertsil ODS-3 (GL Science, Tokyo) was used for HPLC analysis. All other chemicals were reagent grade and obtained from commercial sources.

6.2.3. Construction of recombinant plasmids and *E. coli* strains

The gene encoding TePpk (accession no.: BA000039, protein id: BAC07766.1) was amplified by polymerase chain reaction using KOD plus DNA polymerase (Toyobo, Osaka) with an LIC adapter sequence or *Nde*I and *Eco*RI restriction sites upstream and downstream of *Teppk*, respectively. The amplified DNA fragment with the LIC adapter sequence was introduced into a pET 30 Xa / LIC vector on the basis of the instructions provided by the manufacturer. This recombinant plasmid was named pTePpk and used to produce N-teminal His×6-tagged TePpk. The amplified DNA fragment with restriction sites was digested by *Nde*I and *Eco*RI, and subsequently ligated to a pET 21a (+) vector using DNA ligation kit ver. 2.1 (Takara Bio). This recombinant plasmid was named pPPK and used to produce native TePpk. The recombinant plasmid harboring *Tmddl* was constructed as pPPK except that the *Eco*RI restriction site was altered to the *Hind*III restriction site, and the plasmid was named pDDL. The plasmid pPPK-DDL that has both *Teppk* and *Tmddl* was constructed as follows. A DNA fragment of *Tmddl* including a T7 promoter and a T7 terminator was amplified by PCR using pDDL as a template with an *Sph*I restriction site at 5'- and

3'-termini, and subsequently introduced into the *Sph*I restriction site on pPPK. All recombinant plasmids were introduced into *E. coli* JM 109, and the sequence of each gene was confirmed after plasmid isolation. Subsequently, the sequenced plasmids were introduced into *E. coli* BL21(DE3) for gene expression. The recombinant *E. coli* BL21(DE3) producing N-teminal His×6-tagged TmDdl (*E. coli* BL21(DE3) / pTmDdl) was prepared as described previously (22).

6.2.4. Purification procedure of His×6 tagged protein

E. coli BL21(DE3)/pTePpk or pTmDdl was harvested at 3000 × *g* for 10 min. The cell pellet was treated with 5 ml of BugBuster protein extraction reagent (Merck) for 20 min, and then centrifuged at 16,000 × *g* for 30 min. The supernatant was collected as a cell-free extract. The cell-free extract was applied onto a HisTrap HP 1-ml column equilibrated with 50 mM Tris-HCl (pH 8.0), 500 mM NaCl and 10 mM imidazole, and the column was washed with 10 column volumes of the same buffer. The His×6-tagged protein was eluted with a linear gradient with 50 mM Tris-HCl (pH 8.0), 500 mM NaCl and 500 mM imidazole and desalted on a PD-10 column equilibrated with 50 mM Tris-HCl (pH 8.0). The desalted His×6-tagged protein was applied onto a HiTrap Q HP 1-ml column equibrated with 50 mM Tris-HCl (pH 8.0), and the column was washed with 10 column volumes of the same buffer. The His×6-tagged protein was eluted with a linear gradient with 50 mM Tris-HCl (pH 8.0), and 500 mM NaCl and desalted on a PD-10 column equilibrated with 50 mM Tris-HCl (pH 8.0) again.

6.2.5. Enzyme assay

Polyphosphate kinase activity was measured as ATP synthesizing activity from ADP and polyphosphate. The basal reaction mixture consisted of 100 μg/ml of

purified TePpk, 1 mM ADP, 1 mM $PolyP_3$, $PolyP_4$ or $PolyP_{12-13}$, 10 mM $MgSO_4$ and 50 mM Tris-HCl (pH 8.0). After incubation, the reaction was terminated by adding 5% (w/v) trichloroacetate (final conc.) and the amount of synthesized ATP was quantified by HPLC. One unit of the enzyme activity was defined as the amount of enzyme that synthesized 1 μmol of ATP per min at 60ºC using $PolyP_4$ as a phosphate donor.

D-Alanine-D-alanine ligase activity was measured as described previously (12), and one unit of the enzyme activity was defined as the amount of enzyme that synthesized 1 μmol of D-alanyl-D-alanine per min at 60ºC.

The reaction mixture for an ATP regeneration system coupled with D-alanyl-D-alanine synthesis consisted of 100 μg/ml of each purified TePpk (0.032 U/ml) and TmDdl (0.55 U/ml), 10 mM D-alanine, 0.005 to 5 mM ATP, 1 mM $PolyP_3$, $PolyP_4$ or $PolyP_{12-13}$, 10 mM $MgSO_4$ and 50 mM Tris-HCl (pH 8.0). The reaction was terminated by adding 5% (w/v) trichloroacetate (final conc.) and the amount of synthesized D-alanyl-D-alanine was quantified by HPLC.

6.2.6. Resting cell reaction

E. coli BL21(DE3)/pDDL or pPPK-DDL was cultivated as described above. The cells were harvested at 3000 × *g* for 10 min and washed twice with 50 mM Tris-HCl (pH 8.0). The basal reaction mixture consisted of resting cells (final $O.D._{660}$ = 10), 50 mM D-alanine, 5 mM $PolyP_{12-13}$, 10 mM $MgSO_4$, and 50 mM Tris-HCl (pH 8.0). After the reaction, the cells were removed by centrifugation and the amount of D-alanyl-D-alanine in the supernatant was quantified by HPLC

6.2.7. HPLC analysis

ATP and ADP were measured using an HPLC system equipped with an Inertsil

ODS-3 column equilibrated with 0.1 M Na_2HPO_4 (pH 6.0 by phosphate) and an isocratic elution was used. The flow rate was maintained at 1.0 ml/min, and the column temperature, at 40°C. After separation, UV absorption was monitored at 260 nm.

D-Alanyl-D-alanine was derivetized with FDAA (22) and measured by HPLC equipped with a WH-C18A column. A sample (100 μl) was mixed with 50 μl of 0.5% (w/v) FDAA solution in acetone and 40 μl of 0.5 M $NaHCO_3$ and incubated at 40ºC for 60 min. After deriverization, 40 μl of 0.1 N HCl was added to terminate the reaction and the FDAA-derivetized compounds were subjected to column chromatography. A gradient elution of solvent A (50 mM KH_2PO_4 [pH 2.7 by phosphate]:acetonitrile:methanol=18:1:1) and solvent B (50 mM KH_2PO_4 [pH 2.7 by phosphate]:acetonitrile:methanol=12:7:1) was used with the following program: 0 to 24 min; a linear increase in solvent B to A:B of 55:45, held up to 30 min, 30 to 50 min; a linear increase in solvent B to A:B of 0:100, held up to 55 min. After each gradient elution, the column was washed with solvent C (water: tetrahydrofuran; acetonitrile=1:1:3) for 7 min and re-equilibrated with solvent A for 20 min. The flow rate was maintained at 0.5 ml/min, and the column temperature, at 40°C. After separation, UV absorption was monitored at 340 nm.

6.3. Results

6.3.1. Characterization of His×6 tagged TePpk

TePpk consisted of 2238 bp and annotated as a polyphosphate kinase in a database at DNA Data Bank of Japan. Then, the gene encoding TePpk was cloned and expressed in the recombinant *E. coli* to confirm and characterize the polyphosphate kinase activity of TePpk. His×6-tagged TePpk was successfully produced in the

recombinant *E. coli* BL21(DE3)/pTePpk, although an inclusion body was partially formed. Through two-step column chromatography, His×6-tagged TePpk was purified to homogeneity (TABLE 6.1). The purified enzyme showed polyphosphate kinase activity and synthesized ATP from ADP and $polyP_4$. However, TePpk showed no activity for synthesizing ATP from AMP; therefore, it was confirmed that TePpk catalyzes restricted phosphate transfer among ATP, ADP and polyphosphate.

TABLE 6.1 Purification procedure of recombinant Hisx6 tagged TePpk

Purification step	Total protein (mg)	Total activity (μmol/min)	Specific activity (nmol/min/mg)	Purificatior (fold)	Yield (%)
Cell-free extract	1.39	20.2940	14.6	1.00	100
HisTrap HP	0.302	23.3144	77.2	5.29	21.7
HiTrap Q HP	0.0655	20.7635	317	21.7	4.71

The temperature and pH dependences of TePpk are shown in Fig. 6.1. TePpk was stable and showed a high activity at pH 6 to 8, and this pH dependence was similar to that of polyphosphate kinase from *E. coli*, which was utilized in an ATP regeneration system previously (23). On the other hand, TePpk showed a high thermostability up to 70ºC and a high ATP synthesizing activity at 60ºC. It was previously reported that polyphosphate kinase from *E. coli* loses 27% or 93% of its activity at 55ºC or 60ºC, respectively (23). Therefore, it was revealed that TePpk shows a much higher thermostability than that of polyphosphate kinase from *E. coli*, and expected that a thermostable ATP regeneration system will be established utilizing TePpk. In addition, this was the first report concerning the thermostable polyphosphate kinase.

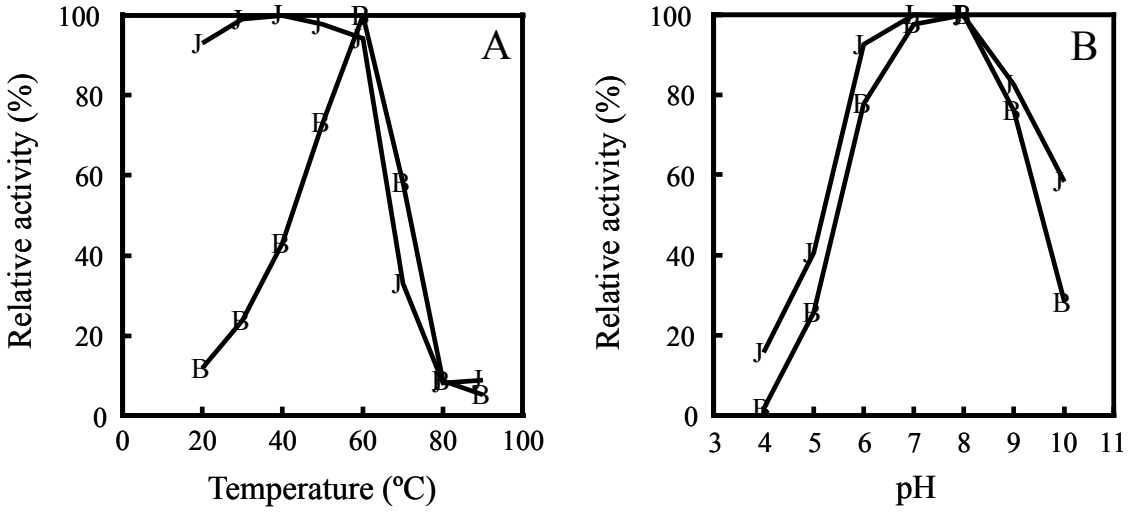

FIG. 6.1. Temperature (A) and pH (B) dependency of TePpk. Temperature (A) and pH (B) dependency of TePpk. (A) The enzyme solution was incubated for 15 min at several temperatures and residual ATP synthesizing activity was measured to determine thermal stability (circles). The reaction was carried out at several temperatures and optimal temperature was determined (squares). (B) The enzyme solution was incubated for 24 h at several pH values and residual ATP synthesizing activity was measured to determine pH stability (circles). The reaction was carried out at several pH values and optimal pH was determined (squares).

Since the optimal reaction conditions were determined to be a temperature of 60ºC and a pH value of 8, the effects of cofactors were investigated for further characterization. Magnesium ions are commonly required by ATP-relating enzymes; in addition, it was reported that the ATP synthesizing activity of polyphosphate kinase from *E. coli* is increased by 14-fold by adding ammonium sulfate (24). In the case of TePpk, the optimal concentration of Mg^{2+} was 0.5 mM, and the ATP synthesizing activity was markedly decreased below 0.5 mM. On the other hand, the excess concentration of Mg^{2+} did not decrease the activity, and 70% of the activity remained even when 10 mM Mg^{2+} was added (data not shown). Despite the previous report, the ATP synthesizing acitivity of TePpk was not increased by adding $(NH_4)_2SO_4$. Therefore, it was revealed that TePpk requires only Mg^{2+} (above 0.5 mM) as a cofactor. This characteristic was suitable for establishing a simple and widely usable ATP regeneration system.

In an ATP regeneration system, an adenosine scaffold is recycled and a large amount of ATP or ADP is not required. However, polyphosphate, as a phosphate donor, is consumed for ATP regeneration. Therefore, a large amount of polyphosphate (as phosphate) is required to achieve a high turnover of ATP regeneration. As shown in Fig. 6.2A, TePpk preferred $PolyP_4$ as a phosphate donor and showed 26% activity against $PolyP_4$ when $PolyP_{12\text{-}13}$ was used. It was also revealed that TePpk cannot utilize $PolyP_3$ as a phosphate donor. Therefore, $PolyP_4$ was preferred by TePpk but one molar $PolyP_4$ could be used to regenerate only one molar ATP. On the other hand, $PolyP_{12\text{-}13}$ was less preferred but capable of regenerating 9 to 10 molar ATP from one molar $PolyP_{12\text{-}13}$. The substrate inhibitions of both $PolyP_4$ and $PolyP_{12\text{-}13}$ were observed (Fig. 6.2B); however, the inhibition level of $PolyP_{12\text{-}13}$ was almost the same above 2 mM, whereas that of $PolyP_4$ was gradually increased. Thereby, it was considered that $PolyP_{12\text{-}13}$ is more preferable as a phosphate donor for an ATP regeneration system utilizing TePpk.

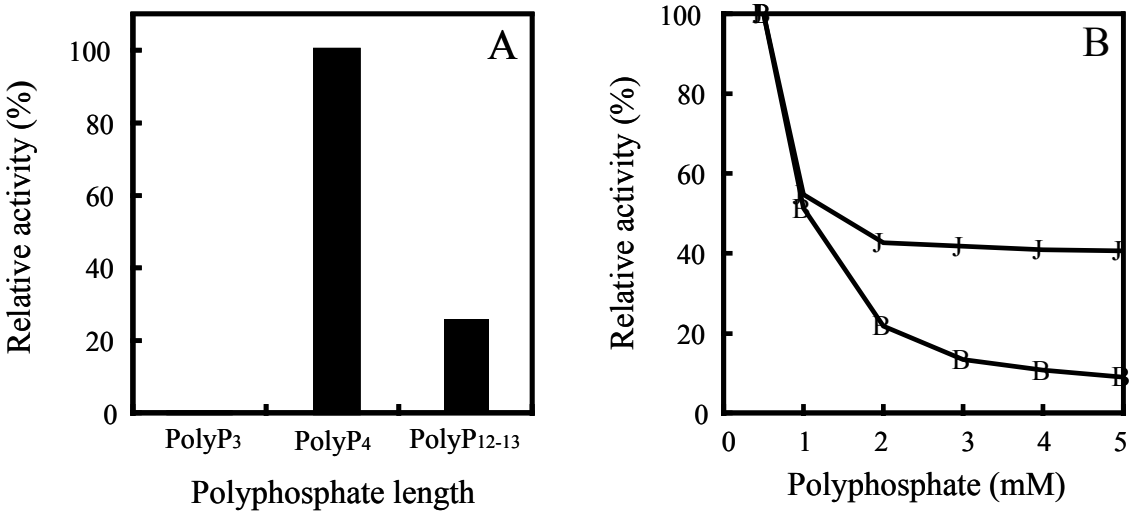

FIG. 6.2. Effects of chain length (A) and concentrations (B) of polyphosphate for ATP synthesizing activity of TePpk. Several concentrations of polyphosphate with three different chain lengths were added to the basal reaction mixture described in Materials and methods, and the reaction was performed at 60ºC for 30 min. Circles and squares indicate the effects of $PolyP_{12\text{-}13}$ and $PolyP_4$, respectively.

6.3.2. Thermostable ATP regeneration system coupled D-alanyl-D-alanine synthesis by purified enzymes

The thermostable ATP regeneration system was then applied to D-alanyl-D-alanine synthesis by TmDdl. The reaction was carried out from 10 mM initial D-alanine at 60ºC, and at least 5 mM ATP was required to achieve a 100% (mol/mol) theoretical yield if there was no ATP regeneration. In the reaction with only TmDdl, the synthesized D-alanyl-D-alanine was markedly reduced with a decrease in initial ATP amount, and D-alanyl-D-alanine was not detected in the reaction with an initial ATP amount below 0.05 mM (Fig. 6.3). On the other hand, in the reaction coupled with the thermostable ATP regeneration system, a marked amount of D-alanyl-D-alanine was synthesized regardless of initial ATP amount. In addition, the yield of D-alanyl-D-alanine was not decreased in the reactions with the initial ATP amounts of 0.5 and 0.05 mM that were below the *K*m value of TmDdl for ATP (0.84 mM). In particular, using $PolyP_{12\text{-}13}$ as a phosphate donor, the initial ATP amount could be reduced to 1% (0.05 mM) of the theoretically required amount to achieve a high yield, the same as when a sufficient amount of ATP (5 mM) was added. Moreover, in the reaction with an initial ATP amount of 0.005 mM, the calculated turnover of ATP regeneration was approximately 570 times.

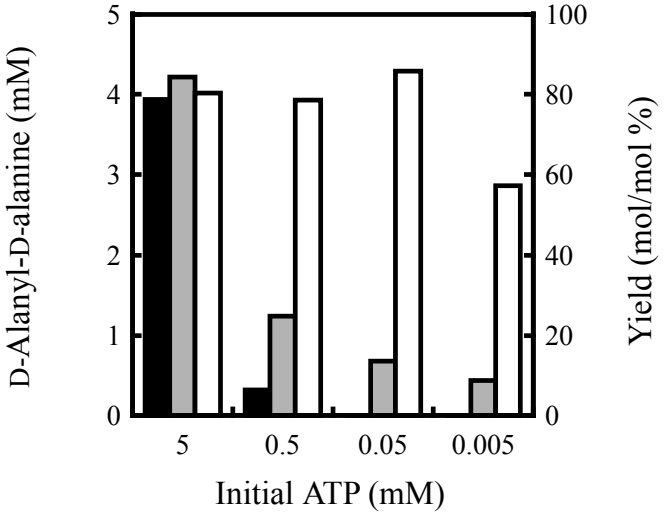

FIG. 6.3. D-Alanyl-D-alanine synthesis coupled with thermostable ATP regeneration system. The reaction mixtures containing TmDdl (closed bar), TmDdl and TePpk with $PolyP_4$ (shaded bar) or TmDdl and TePpk with $PolyP_{12\text{-}13}$ (open bar) were incubated with several initial ATP amounts at 60ºC for 20 h. The amount of D-alanyl-D-alanine when most initial D-alanine was converted was defined as 100% of the yield.

6.3.3. D-Alanyl-D-alanine synthesis using resting cells

To enhance the availability of the thermostable ATP regeneration system and an effective D-amino acid dipeptide synthesis method, the enzymatic coupling reaction of TePpk and TmDdl was applied into the resting cell reaction. This reaction should be performed at 60ºC; however, there were few reports concerning resting cell reaction using *E. coli* cells at a high temperature. Then, the availability of *E. coli* cells was examined at a high temperature. When the cell suspension of *E. coli* BL21 (DE3)/pDDL (initial $O.D._{660}$ = 10) was incubated at 37 or 60ºC for 24 h, the values of $O.D._{660}$ shifted to 5.5 or 6.2, respectively. It was considered that the optical density at 660 nm is reduced by cell lysis. However, the cell lysis level occurring at 60ºC was the same as that at 37ºC. In addition, the result of SDS-PAGE analysis of the supernatant prepared from the cell suspension incubated for 24 h showed that the amounts of TmDdl leaking to the supernatant at 37ºC and 60ºC are almost the same

(data not shown). Although enzymes from the *E. coli* host must have been inactivated, it was suggested that *E. coli* cells can be used even at 60ºC as an enzyme vessel. In the reaction with the resting cells of *E. coli* BL21(DE3)/pPPK-DDL, which showed both TePpk and TmDdl activities, 22.9 mM D-alanyl-D-alanine was successfully synthesized accompanying the regeneration of only 0.05 mM adenosine scaffold, whereas only 2.7 mM D-alanyl-D-alanine was synthesized using *E. coli* BL21(DE3)/pDDL under the same reaction condition. Therefore, it was confirmed that the coupling reaction with TmDdl and TePpk certainly occurs even with the resting cells. In the view of substrate and product permeabilities, it was supposed that a high reaction temperature damages the *E. coli* cells and increases the permeability similarly to a treatment with an organic solvent. Therefore, it was suggested that the resting cell reaction at a high temperature omits the enzyme purification step and increases material permeability without a particular cell treatment.

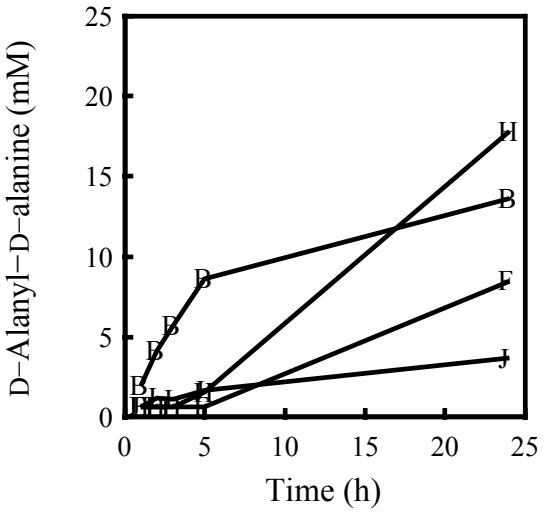

FIG. 6.4. Time course of D-alanyl-D-alanine synthesis with several concentrations of PolyP$_{12\text{-}13}$ in the resting cell reaction. The resting cell reaction using *E. coli* BL21(DE3)/pPPK-DDL cells was carried out with 0.5 (circles), 5 (squares), 10 (triangles), and 20 mM (diamonds) PolyP$_{12\text{-}13}$ at 60ºC. Other reaction conditions were described in Materials and methods.

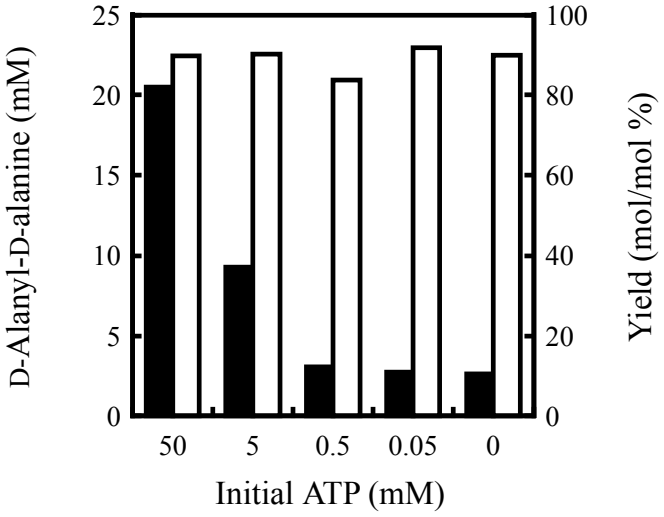

FIG. 6.5. ATP concentration required for D-alanine-D-alanine synthesis in resting cell reaction. The resting cell reaction using *E. coli* BL21(DE3)/pDDL (closed) or pPPK-DDL (open) cells was carried out with several initial ATP concentrations at 60ºC for 24 h. The reaction was carried out in the optimized reaction mixture consisting of resting cells (final O.D. 660 = 10), 50 mM D-alanine, 10 mM $PolyP_{12\text{-}13}$, 10 mM $MgSO_4$, and 50 mM Tris-HCl (pH 8.0). The amount of D-alanyl-D-alanine when most initial D-alanine was converted was defined as 100% of the yield.

6.4. Discussion

In this chapter, an effective synthesis of D-alanyl-D-alanine with the thermostable ATP regeneration system was demonstrated. Since D-alanyl-D-alanine was effectively synthesized in the coupling reaction, it was confirmed that the thermostable ATP regeneration system was certainly operated at the high temperature, and TmDdl coupled with TePpK could successfully synthesize D-alanyl-D-alanine with a few amount of initial ATP. Comparing with TePpk, ATP could not be regenerated at 60ºC with polyphosphate kinase from *E. coli* (data not shown), therefore, the thermostable ATP regeneration system was achieved due to the thermostability of TePpk.

As shown in FIG 6.2, an excess amount of polyphosphate inhibited the ATP synthesizing activity of TePpk, however, more polyphosphate was required to achieve more turnover of ATP regeneration. Therefore, in the resting cell reaction, the fastest

reaction velocity for the D-alanyl-D-alanine synthesis was achieved with 5 mM $PolyP_{12\text{-}13}$, however, D-alanyl-D-alanine was synthesized in the maximum yield with 10 mM $PolyP_{12\text{-}13}$ (FIG. 6.4). The inhibition of TePpk activity with 5 mM $PolyP_{12\text{-}13}$ was less than that with 10 mM $PolyP_{12\text{-}13}$, thus, the fastest reaction velocity would be achieved with 5 mM $PolyP_{12\text{-}13}$. On the other hand, $PolyP_{12\text{-}13}$ would be degraded by heat or residual other enzyme activities in the reaction mixture, and 5 mM $PolyP_{12\text{-}13}$ was not capable to regenerate the sufficient amount of ATP. Taken together, the optimal concentration of $PolyP_{12\text{-}13}$ was determined to be 10 mM in this reaction.

In the resting cell reaction, it was surprising finding that D-alanyl-D-alanine could be synthesized without addition of initial ATP. In this reaction, it was considered that the adenosine scaffold remained in the cell was regenerated by TePpk, and the high yield synthesis of D-alanyl-D-alanie was achieved. As also mentioned in chapter 3, it was suggested that ATP could be regenerated using ATP scaffold in the cell when using resting cell. Therefore, this fact is considered to be an important advantage of utilizing resting cell not only D-amino acid dipeptide synthesis but also other enzymatic reaction required ATP.

Besides, TmDdl recognized not only D-alanine but also D-serine, D-cystein, D-threonine and other D-amino acids as substrates. Therefore, it was considered that various kinds of D-amino acid dipeptides could be effectively synthesized in this process. Moreover, this resting cell reaction system for thermostable ATP regeneration was not restricted only for the synthesis of D-amino acid dipeptides and it was expected that this system could be applied to other reactions with ATP-dependent thermostable enzymes.

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Chapter 7

Summary and Conclusions

In this thesis, the author described the development of effective production process for D-amino acid dipeptide. The summary of this thesis is as follows.

In chapter 1, the overview of enzymes capable to synthesize D-amino acid peptide was described. These enzymes require D-amino acid esters or amides as starting materials and most of these enzymes show hydrolyzing activity toward the synthesized D-amino acid peptide resulting low yield. Therefore, another enzyme that utilizes free D-amino acid and have no hydrolyzing activity was desired to develop the effective production process. Then, D-alanine-D-alanine ligase (Ddl) was focused as a useful enzyme for producing D-amino acid dipeptide. The overview of Ddl and its mutant was described and the possibility of the effective production utilizing Ddl was suggested. Based on these review, the author clarified the objective of this thesis.

In chapter 2, the author described characteristics of several Ddls and synthesis of D-amino acid dipeptide utilizing Ddl. The genes encoding Ddls from *Escherichia coli* K12 (EcDdlB), *Oceanobacillus iheyensis* JCM 11309 (OiDdl), *Synechocystis* sp. PCC 6803 (SsDdl) and *Thermotoga maritima* ATCC 43589 (TmDdl), the genomic DNA sequences of which have been determined, were cloned and the substrate specificities of these recombinant Ddls were investigated. Although OiDdl had high substrate specificity for D-alanine; EcDdlB, SsDdl and TmDdl showed broad substrate specificities for D-serine, D-threonine, D-cysteine and glycine, in addition to D-alanine. On the basis of the substrate specificity, D-amino acid dipeptides were successfully

produced utilizing EcDdlB. Beside, TmDdl was expected as a thermostable enzyme suitable for industrial production process.

In chapter 3, coupling reaction of EcDdlB and broad specificity amino acid racemase (BAR) was performed in order to supply D-amino acid from inexpensive L-amino acid. The gene encoding BAR was cloned and overexpressed in *E. coli* BL 21(DE3)/pLysS. Since the recombinant BAR showed broad substrate specificity toward almost all the standard amino acids, it was suggested that BAR was suitable for the coupling reaction. In the coupling reaction with purified EcDdlB and BAR, D-amino acids were supplied from L-amino acids as expected, subsequently D-amino acid dipeptides were successfully synthesized. Besides, ATP synthesis through glycolytic pathway was effectively coupled when the reaction was applied onto resting cell reaction. In this resting cell reaction, the effective D-amino acid production that requires only L-amino acid and glucose as starting material was achieved.

In chapter 4, random mutagenesis on *bar* was performed to obtain mutant BAR derivatives with more broad substrate specificity toward poor substrates such as tryptophan. Five positive mutants were isolated after the two-step screening of the randomly mutated BAR. After the determination of the amino acid substitutions in these mutants, it was suggested that the substitutions at Y396 and I384 increased the tryptophan specific racemization activity and the racemization activity for overall amino acids, respectively. Among the positive mutants, I384M mutant BAR showed the highest activity for tryptophan. Besides, the activity of I384M mutant BAR was markedly increased toward other amino acid. Thereby, it was expected that I384M mutant BAR would contribute to the effective supply of D-amino acid not only in the reaction with Ddl but also in other reaction synthesizing D-amino acid derivatives.

In chapter 5, the author described the characteristics of TmDdl obtained from

thermophilic bacteria, *Thermotoga maritima* ATCC 43589. TmDdl was thermostable between 10ºC to 90ºC, and this thermostability was expected to be preferable in industrial production process as for long term storage and easy handling. In the reaction at 60ºC, TmDdl showed more broad substrate specificity toward 15 D-amino acids, while only D-alanine, D-serine, D-cystein, D-threonine, and glycine were recognized as substrate at 37ºC. The CD spectrum of TmDdl at 60ºC slightly differed from that at 37ºC, however, it was considered that the expanded substrate specificity was not due to dynamic conformation change of TmDdl but enhanced the reaction velocity of TmDdl. These preferable characteristics were the result from utilizing the advantage of thermostable enzyme.

In chapter 6, a thermostable ATP regeneration system was established and coupled to D-amino acid dipeptide synthesis with TmDdl. TmDdl showed the broad substrate specificity at high temperature, however, ATP was required for its reaction. One of the methods for an effective ATP supply was the coupling reaction with an ATP regeneration system. However, ATP regeneration systems were consisted of enzymes from mesophile, and hard to operate at high temperature. Therefore, ATP regeneration system that could be used at high temperature was desired to utilize TmDdl for effective production process of D-amino acid dipeptides. In order to establish a thermostable ATP regeneration system, polyphosphate kinase from a thermophile, *Thermosynechococcus elongatus* BP-1 (TePpk) was characterized. TePpk showed thermostability up to 70ºC, therefore, it was considered that a thermostable ATP regeneration system could be established using TePpk. In the coupling reaction with purified TmDdl and TePpk at 60ºC, the amount of ATP required for D-alanyl-D-alanine synthesis could be reduced to 1% of the theoretical amount required when there was no ATP regeneration. When the coupling reaction was applied onto resting cell reaction,

ATP was regenerated from adenosine scaffold in the cell, and D-alanyl-D-alanine was successfully synthesized in the maximum yield of 80% (mol / mol) without addition of ATP. Thus, an effective synthesis of D-amino acid dipepitide was achieved using the thermostable ATP regeneration system.

In the biosynthesis of D-amino acid peptide, the maximum yield has been 60% reported so far (1-7). In contrast, it was demonstrated that Ddl could synthesize D-amino acid dipeptide over 80% yields due to its one-directional reaction following hydrolyzing ATP. Of course, requirement of expensive ATP is severe problem, therefore ATP dependent enzymes are regarded as unsuitable in industrial processes. However, as described in this thesis, the effective supply of ATP was achieved by utilizing cell's own function and enzymatic ATP regeneration system. Thereby, D-amino acid dipeptide synthesis with Ddl is no longer unsuitable but appropriate process for industrial production. In parallel with Ddl, Tabata *et al.* reported L-amino acid α–ligase (LAL) that catalyzes the synthesis of L-form dipeptide by ligating free L-amino acid (8), and established an excellent production process (9). D- and L-form dipeptide synthesis utilizing amino acid ligases including Ddl and LAL is considered as the first step for the effective and industrial bioproduction of peptide. Among amino acid ligases, it is known that some glutathione synthetases show altered substrate specificity and synthesize tripeptide (10), and some mutant Ddl recognize 2-oxo acids as C-terminal substrate. Therefore, not only D,L-amino acid dipeptides but also longer peptides and peptide relating compounds *i.e.* depsipeptides would be synthesized utilizing various amino acid ligases in future. Based on the technology for the effective production of peptides, it is expected that more and more useful function of peptides are developed and they would contribute to the plentiful human life.

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Acknowledgment

The studies in this thesis were accomplished under the direction of Professor Kuniki Kino at Waseda University in Tokyo during the period of 2002 to 2007. I would like to express my deepest gratitude to Professor Kuniki Kino for his continuous support and encouragement. I also would like to express my gratitude to Professor Kohtaro Kirimura and Professor Hiroyuki Niside for their continuous support and encouragement.

Through the studies in this thesis, I am deeply grateful to Dr. Makoto Yagasaki at Kyowa Hakko Kogyo Co., Ltd. for his many support and encouragement, and Kazusa DNA Research Institute for providing the genomic DNAs of *Synechocystis* sp. PCC6803 and *Thermosynachococcus elongatus* BP-1.

It would have been impossible to complete this thesis without the support and encouragement of many people. I am particularly grateful to Assistant Professor Yoshitaka Ishii, Dr. Toshiki Furuya, Dr. Toshiyuki Sato, Dr. Takako Murakami, Mr. Kiyotake Kamigaki, and Mr. Toshinobu Arai.

Finally, the studies in this thesis were performed with my 16 collaborators, and I wish to acknowledge them; Ms. Mariko Yoneyama, Mr. Yusuke Masuda, Mr. Ryotaro Hara, Mr. Yuji Nakazawa, Mr. Yohichi Kotanaka, Ms. Shoko Kuratsu, Mr. Masahiro Kokubo, Ms. Ryoko Satake, Ms. Momoko Takagaki, Ms. Ai Nozawa, Ms. Ikumi Kurihara, Mr. Atsushi Noguchi, Ms. Mai Saito, Mr. Hisashi Osanai, Mr. Yuji Takazawa, and Mr. Kohsuke Hidaka.

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研究概要

ペプチドは抗菌物質やシグナル分子として自然界に見出され、その多様な機能から医薬品原料や機能性食品など幅広い分野で用途開発が進められている。また近年の化学的なペプチド合成技術の進展と分析技術のハイスループット化は、多様なペプチドの創出と迅速な機能解析を可能としている。このような技術革新を基盤として、タンパク質性のアミノ酸からなる天然型ペプチドに抗菌活性や降圧活性など有用な機能が数多く見出されている。一方、ペプチドの機能は構成するアミノ酸の特性に由来するため、非天然型を含む多様なアミノ酸で構成されるペプチドを合成することができれば、さらに機能の多様性を拡張できるものと期待される。しかし、D-アミノ酸など非天然型アミノ酸を含有するペプチドに関しては、天然型ペプチドと同様な効率的合成法がないために用途開発研究が停滞しており、機能開発研究の発展にはより効率的な合成法が必須となっている。

本研究では、D-アミノ酸含有ペプチドの効率的合成プロセスを構築するために、微生物が有するアミノ酸リガーゼである D-アラニン-D-アラニンリガーゼ（Ddl）に着目した。Ddl は細菌のペプチドグリカンの生合成に関わる酵素であり、遊離の D-アラニン 2 分子を連結してジペプチドを合成する。また、一部の微生物が有する変異 Ddl には D-アラニン以外に D-セリンや D-乳酸の結合活性が報告されている。このことから、多様な微生物の中にはより広範な基質特異性を有する Ddl が存在していると期待され、このような Ddl を利用することで基質の修飾を必要としない簡便で効率的な D-アミノ酸ジペプチドの 1 段階合成プロセスが構築できるものと考えた。しかし、広範な基質特異性を有する Ddl の報告はこれまでにない。また、Ddl によるジペプチド合成反応には高価な ATP が必要となる。こうした状況を踏まえ、本論文では、Ddl の特性解析によって見出した広範な基質特異性を有する Ddl とそれを利用した効率的な D-アミノ酸ジペプチド合成プロセスについて論じた。

本論文は 7 章より構成される。以下にその概要を述べる。

第 1 章では、既存のペプチド合成法の中でも D-アミノ酸ペプチド合成に関わるバイオプロセスを中心に、現在の合成法の概要と課題を挙げた。さらに、本研究の中心となる酵素である Ddl に関連する生理学的知見を概説し、本研究の着想に至った経緯を論じた。

第 2 章では、数種類の微生物から取得した異なる Ddl ホモログの機能解析とその結果について説明を加えた。多様な D-アミノ酸ジペプチド合成のためには広範な基質特異性を有する Ddl が必要となる。そこで、ゲノム情報を基に多様性が期待される *Escherichia coli* K-12（EcDdlB）、*Synechocystis* sp. PCC 6803（SsDdl）、*Oceanobacillus iheyensis* JCM 11309（OiDdl）、*Thermotoga maritima* ATCC 43589（TmDdl）由来の 4 種類の Ddl 遺伝子をクローニングし、組換え酵素の基質特異性を検証した。OiDdl は D-アラニンのみに特異性を示す酵素であったが、その一方で、EcDdlB、SsDdl、TmDdl は D-アラニンに加え、D-セリン、D-システイン、D-スレオニン、グリシンに対しても活性を示すことを明らかにした。とくに常温で高活性を示す EcDdlB を利用した D-アミノ酸ジペプチド合成では、遊離の D-アミノ酸から 1 段階の酵素反応で最大 77%(mol/mol)の収率を達成し、D-アミノ酸ジペプチド合成における Ddl の有用性を示した。また、TmDdl は好熱性菌由来の酵素であり、工業利用に有用な耐熱性と安定性が期待された。

第 3 章では、安価な L-アミノ酸を原料とするために、アミノ酸ラセマーゼを Ddl と共役させたプロセスについて論じた。この共役プロセスには広範な基質特異性を有するアミノ酸ラセマーゼが必要であることから、*Pseudomonas putida* IFO 12996 由来低基質特異性アミノ酸ラセマーゼ（BAR）に着目し、当該酵素遺伝子のクローニングを行った。取得した組換え酵素の特性について詳細に検討したところ、BAR はほぼ全ての標準アミノ酸を基質とし得る広範な基質特異性を有することを明らかにした。L-アミノ酸から D-アミノ酸の供給を可能とする BAR と EcDdlB との共役反応によって、安価な原料からの D-アミノ酸ジペプチド合成を達成した。さらに、本共役反応を菌体反応プロセスに応用し、菌体が元来有するグルコースからの解糖系に依存した ATP 再生能を利用することを考えた。そこで、EcDdlB と BAR を高発現する組換え大腸菌を造成し、D-アミノ酸

ジペプチド合成に適した培養条件を検討した。最適条件下では、外部からの L-アラニンとグルコースを添加した 27 時間の反応において D-アラニル-D-アラニンを収率 74%(mol/mol)で合成することができた。このように菌体の ATP 再生能の適切な利用により、効率的な D-アミノ酸ジペプチド合成プロセスの確立に成功した。

第 4 章では、前章で L-アミノ酸からの D-アミノ酸供給に用いた BAR の基質特異性をさらに拡張するために、BAR が極めて微弱な活性しか示さない芳香族アミノ酸、中でもトリプトファンに対する活性が向上した BAR 変異体の創製を試みた。BAR の立体構造は決定されていないことから、酵素改変には遺伝子に人工的にランダムな変異を導入して、目的の候補変異体をスクリーニングする進化分子工学的手法が有効であると考えた。そこで、膨大なランダム変異ライブラリーから目的とする候補変異酵素を取得するために、トリプトファン栄養要求性大腸菌の生育を指標とした効率的なスクリーニング系を構築した。本スクリーニング系を用いて 30,000 クローンの変異ライブラリーから、80 個の候補変異体を選別した。さらに、これらの候補変異体のトリプトファンに対するラセミ化活性を比色分析により定量することで、最終的にトリプトファンのラセミ化活性が向上した 5 種類の変異酵素を取得した。変異酵素の解析から、BAR の 384 位のイソロイシン残基と 396 位のチロシン残基が BAR のトリプトファンに対するラセミ化活性の向上に寄与することを明らかにした。さらに、分子モデリング解析から、両変異が BAR の補酵素であるピリドキサール 5'-リン酸の結合に関与している可能性を示した。最も高活性を示した 384 位のメチオニン置換体(I384M BAR)は野生型酵素の 20 倍のトリプトファンラセミ化活性を示し、さらに、他のアミノ酸に対しても顕著に活性が向上していた。I384M 変異体の創製により、トリプトファンを含め多くのアミノ酸のラセミ化が達成され、反応プロセスへの効率的な D-アミノ酸供給が可能になるものと期待された。

第 5 章では、第 2 章で見出した有用な Ddl の中でも耐熱性が期待される好熱性細菌 *T. maritima* ATCC 43589 由来 TmDdl の酵素諸性質と基質特異性について論じた。耐熱性酵素は、常温においても安定であり長期保存が可能なことや取

り扱いの簡便さなどから、産業用酵素に適した特性を有する。本研究で取得した TmDdl も 10°C から 90°C の範囲において安定であった。さらに、TmDdl の反応速度は温度の上昇に伴って顕著に増大することとその基質特異性が拡張することを見出した。TmDdl は、常温では D-アラニン、D-セリン、D-システイン、D-スレオニン、グリシンを基質としたが、反応温度を 60°C に上昇させると、これらの D-アミノ酸に加えて脂肪族 D-アミノ酸、一部の塩基性 D-アミノ酸や芳香族アミノ酸である D-フェニルアラニンも基質とした。37°C と 60°C における CD スペクトルに大きな差異はなく、酵素分子のダイナミックな構造変化を伴っていないことが示唆され、基質特異性の拡張は高温における TmDdl の反応速度の向上に起因するものと推察した。TmDdl の 60°C における広範な基質特異性は他の Ddl と比較しても特長的なものであり、耐熱性酵素の利点を活用することで見出すことのできた酵素の有用な特性である。

第 6 章では、TmDdl が広範な基質特異性を示す高温域における効率的な ATP 供給方法を検討した。第 3 章で述べたように、解糖系に依存した ATP 再生は常温反応において有効に機能するが、高温反応では酵素の熱失活により解糖系が機能しない。そこで、好熱性細菌 *Thermosynechococcus elongatus* BP-1 由来のポリリン酸キナーゼ（TePpk）に着目し、当該酵素によるポリリン酸をエネルギー源とした耐熱性 ATP 再生系の構築を試みた。大腸菌で組換え酵素として生産させた TePpk は 10°C から 70°C までの広範な温度域で安定な酵素であった。TePpk を組み込んだ耐熱性 ATP 再生系を TmDdl の反応に共役させた 60°C の高温反応条件下では、微量 ATP の添加のみで 80%(mol/mol)を超える高収率での D-アラニル-D-アラニンが合成可能であった。さらに菌体内に微量残存するアデノシン骨格を ATP 再生に利用することを考え、本反応を菌体系にて実施した。その結果、合成収率 85%(mol/mol)を達成し、外部からの ATP 添加を必要としない効率的なプロセスの構築に成功した。

第 7 章では、第 2 章から第 6 章で得られた研究成果を総括し、本研究で用いた Ddl などアミノ酸リガーゼを利用したペプチド合成プロセスの今後の展望について議論した。

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