Review

Ribosome-independent Peptide Synthesis in Nature and Their Application to Dipeptide Production

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While the ribosome system is the major equipment for peptide bond formation in nature, various ribosome-independent peptide-bond forming activities also exist. These activities are not only responsible for the syntheses of specific peptides but also involved in the biosynthesis of cofactors and nucleic acid and in the processes of protein breakdown. In this review, the ribosome-independent activities are overviewed and recent attempts to apply the activities in dipeptide production are introduced.

Key words: peptide bond, nonribosomal peptide synthetase, L-amino acid α-ligase, dipeptide

Introduction

Proteins (and peptides) are major components of organisms and play central roles in living creatures. Since they are configured by peptide bonds, peptide bond is one of the most abundant chemical bond in organisms. Proteins are synthesized by the ribosome system, which is ubiquitously equipped by cellular organisms. The system is very sophisticated and excellent as it can synthesize any sequence of amino acids with marvelous accuracy. While the ribosome system is the major player of peptide bond formation in nature, it is not the only player. Activities other than the ribosome system, which combine amino acids to form peptide bonds, certainly exist. In this article, these ribosome-independent peptide-bond forming activities are reviewed and their application in the production of dipeptides, the simplest peptides.

Existence of peptide bonds in nature

There is no question that the majority

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of peptide bonds in nature belong to protein. However, various compounds other than protein that contain peptide bond(s) also exist.

Purine biosynthesis takes the peptide-bond containing intermediate, 2-[5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamideo]-succinate (1, 2). Coenzyme A, an essential co-factor, contains two peptide bonds (3-5). Folate in organisms is modified by polyglutamate (6). Glutathione is a tripeptide (γ-Glu-Cys-Gly), distributed in a wide range of organisms, and plays a central role in keeping redox balance in the cell (7). Carnosine (β-Ala-His) and anserine (β-Ala-methylhistidine) are dipeptides, found in muscle or brain of mammal, bird, or fish (8, 9). Eubacteria contain short peptides containing D-Ala in their cell surface peptidoglycan (10, 11).

Some kinds of microorganisms have been known to produce polymers build up with amino acid(s); poly-γ-glutamate by *Bacillus* and several other species, ε-poly-lysine by *Streptomyces albulus*, and cyanophicin (co-polymer of Asp and Arg) by *Cyanobacteria* (12). There are numerous bioactive oligo-

peptides and derivatives of oligopeptide produced by microorganisms such as penicillin, vancomycin, and gramicidin S. Dipeptides containing unusual amino acids have also been reported to be produced by microorganisms such as bacilycin (Ala-anticapsin) by *Bacillus subtilis*, bestatin (3-amino-2-hydroxyl-4-phenylbutanoic acidyl - Leu) by *Streptomyces olivoreticuli* (13). Two molecules of amino acids can form two peptide bonds, resulting in a cyclic structure (diketopiperazine). Diketopiperazines are known to be found in microbial metabolite such as albonoursin (a derivative of cyclo (Phe-Leu)) by *Streptomyces noursei* and thaxtomin (a derivative of cyclo (Trp-Phe)) by *Streptomyces acidiscabies* (13).

It should be mentioned that peptide bond formation occurs not only in biosynthesis of some compounds but also in degradation of protein (14). In eukaryotes, proteins with N-terminal primary destabilizing residues are subjected to ligation with ubiquitins followed by degradation mediated by the 26S proteasome complexes. In eubacteria, specific amino acids such as Phe are added to the N-terminal residue of proteins that are degraded via the pathway governed by N-end rule.

These examples show the versatility and widespread importance of peptide bond in organisms. Interestingly, formations of these nonproteinous peptide bonds do not depend on the ribosome system. Specific enzymes are responsible for the formation of peptide bonds, some of which will be introduced in the following section.

Peptide-bond forming activities

To add an amino acid to other amino acid or peptide, the amino acid should be activated. The activation reaction requires ATP

Table   Peptide-bond forming activities in nature

Active intermediate	Carrier	Enzyme or system	Reference
Aminoacyl-AMP	Aminoacyl-tRNA	Ribosomal system	15
		MurM	11
		BppA1	16
		FemXAB	17
		Leucyl/phenylalanyl-tRNA protein transferase	19
	No	NRPS	20, 21
		ACV synthetase	45
		ε-polylysine stnthetase	46
		Pantotanate β-Ala ligase	3, 4
		Phosphopantotanate Cys ligase	5
Aminoacyl-phosphate	No	Folylpolyglutamate synthase	6
		γ-Glutamylcysteine synthetase	22
		Glutathione synthetase	22
		Phosphoribosylaminoimidazole succinocarboxamide synthase	2
		L-Amino acid α-ligase	23
		Cyanophycin synthetase	25
		D-Ala-D-Ala ligase	10
		MurC, D, E, F	11
?	?	AlbC	31

Poly-γ-glutamate synthetase is not included since the reaction mechanism is under discussion.
?: Reaction mechanism of AlbC has not been elucidated.

and two kinds of active intermediates have been known (Table).   One is aminoacyl-AMP, which is formed by the following reaction; amino acid + ATP → aminoacyl-AMP + pyrophosphate (PPi).   The other is aminoacylphosphate, which is formed by the following reaction; amino acid + ATP → aminoacylphosphate + ADP.   Peptide-bond forming activities taking aminoacyl-AMP are represented by the ribosomal system and nonribosomal peptide synthetase. In the ribosome system, an aminoacyl residue is transferred from aminoacyl-AMP to the corresponding t-RNA to form a stable intermediate aminoacyl-tRNA.   In a narrow sense, peptide bond formation in the ribosome system occurs at the peptidyltransfer reaction on rRNA (15). But in this review, peptide-bond forming activity is defined the activity including activation of amino acids.   It should be worthy for noticing that some nonribosomal peptide-bond forming activities also use aminoacyl-tRNA.   On the other hand, glutathione synthase and L-amino acid α-ligase are the examples of the activities via aminoacylphosphate.   Several typical enzymes other than ribosomal system are briefly reviewed below.

Aminoacyl-tRNA dependent nonribosomal peptide-bond forming activities

The peptidoglycan in *Streptococcus pneumoniae* contains "stem peptide" composed of up to five amino acids, Ala-γ-D-Glu-Lys-D-Ala-D-Ala, of which the lysyl residue ε-amino group is substituted by a dipeptide branch of Ala or Ser followed by Ala. MurM is responsible for the addition of Ala or Ser to the stem peptide Lys.   Recently it was revealed that MurM transfers Ala or Ser from Ala-tRNA[Ala] or Ser-tRNA[Ser], respectively, to the stem peptide (11).   BppA1 in *Enterococcus faecalis* and FemXAB in *Staphylococcus aureus* are supposed to catalyze the similar reactions
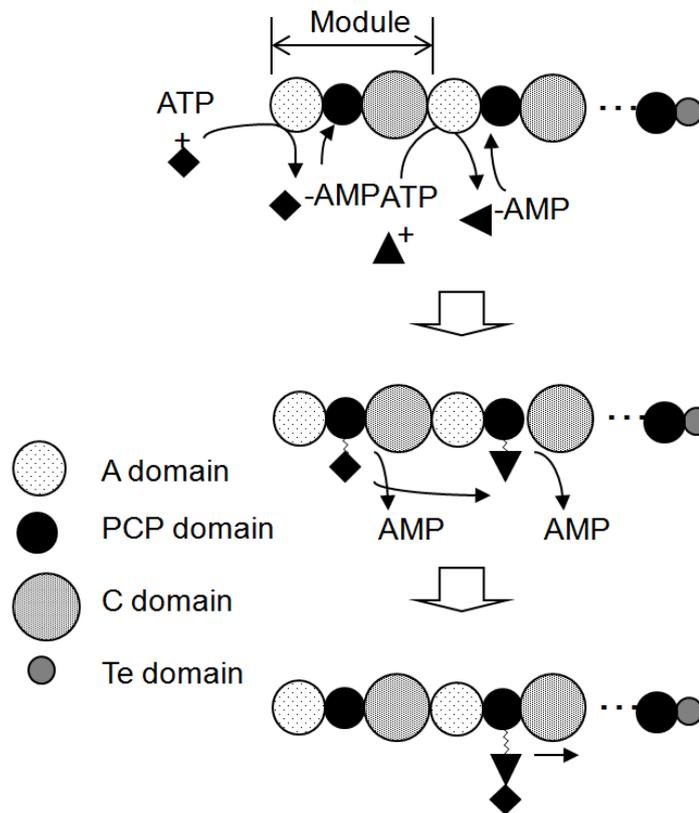

Fig. 1   Schematic view of peptide-forming reaction by NRPS. Diamonds and triangles indicate amino acids.

(16, 17).

In *Escherichia coli*, proteins, which are to be degraded and bearing an N-terminal Arg (or Lys), recruit Phe or Leu to their N-terminal and then enter into N-end rule pathway of proteolysis (18). The recruit is carried out by leucyl/phenylalanyl-tRNA protein transferase. The enzyme transfers Leu and Phe residue from Leu-tRNA^{leu} and Phe-tRNA^{phe}, respectively, to the N-terminal of the target protein (19).

NRPS (Nonribosomal peptide synthetase)

NRPSs are responsible for the syntheses of a wide array of therapeutically important peptides produced by microorganisms such as vancomycin, gramicidin S, and cyclosporine. ACV synthetase, which catalyzes the formation of tripeptide backbone of penicillins and cephalosporins, belongs to NRPS. NRPSs are huge, multifunctional proteins. They are made up of a series of modules, each of which takes charge of adding one amino acid to a growing peptide. Each module contains at least three enzymatic units called domains (Fig. 1). An adenylation domain (A-domain) recognizes the substrate amino acid and activates it as an aminoacyl-AMP. The selectivity-conferring "nonribosomal" code of the A-domain has been determined. The activated amino acid is transferred to 4'-phophopantetheine moiety of the thiolation domain (T-domain) with the release of AMP. Then the adjacent condensation domain (C-domain) catalyzes the formation of the peptide bond. Finally, the thioesterase domain (Te-domain) catalyzes the release of the product peptide from the enzyme protein. NRPS have also been reported to be involved in the synthe-

sis of cyclo-dipeptide. For more details on NRPS, please refer to the comprehensive reviews (20, 21).

γ-Glutamyl-cysteine synthetase and glutathione synthetase

As glutathione is the most abundant non-protein thiol compound widely distributed in organisms, the biosynthesis of the tripeptide have been opened as early as in 1950s. Biosynthesis of glutathione occurs in two steps; Glu + Cys + ATP → γ-Glu-Cys + ADP + Pi and γ-Glu-Cys + Gly + ATP → glutathione + ADP + Pi. The former reaction is catalyzed by γ-glutamyl-cysteine synthetase and the latter one is catalyzed by glutathione synthetase. Substrate specificities of both enzymes are limited to their natural substrates and structural analogs (22).

L-amino acid α-ligase (Lal)

Tabata and coworkers discovered a gene coding for a novel enzyme named L-amino acid α-ligase (Lal) from *Bacillus subtilis*'s genome (23). Lal catalyzes α-dipeptide formation from L-amino acids in the presence of ATP (Fig. 2). It exhibits so wide substrate specificity as 44 kinds of dipeptide can be synthesized, but never reacts with D-amino acids. It never forms tri- or longer peptides. From the amino acid sequence and biochemical data, Lal is considered to belong to ATP-dependent carboxylate-amine/thiol ligase superfamily (24) as well as γ-glutamyl-cyteine synthetase and glutathione stnthetase.

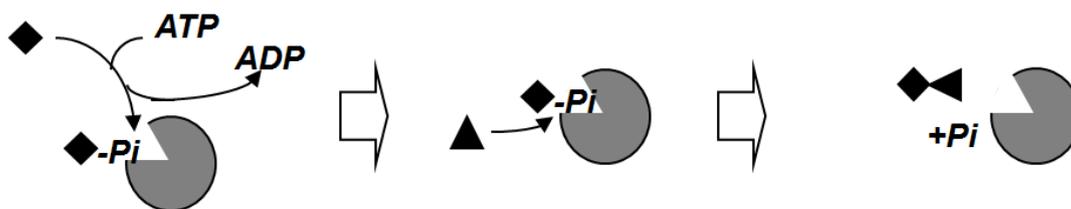## Cyanophysin synthetase and γ-polyglutamate

Fig. 2 Schematic view of peptide-forming reaction by Lal.
Diamonds and triangles indicate amino acids. Partially opened circles indicate Lal.

synthetase

Cyanophycin [multi-Arg-poly(Asp)] is a branched peptide, uniquely found in cyanobacteria. Cyanophycin synthetase, encoded on *cphA*, is responsible for the biosynthesis of the polymer. It was demonstrated that the purified enzyme catalyzed polymer synthesis from Arg, Asp, and a primer polymer concomitant with hydrolysis of ATP to ADP (25). Based on the biochemical data and its amino acid sequence, the enzyme is regarded to take aminoacylphosphate intermediates (25, 26).

Poly-γ-glutamate is produced by several *Bacillus* strains and a few other organisms. The genes governing the synthesis of the polymer have been identified in *B. anthracis* (*capB* and *C*) and *B. subtilis* (*pgsB* and *C*). While biochemical characteristics of the enzymes have also been revealed, argumentation on the active intermediate (aminoacylphosphate or aminoacyl-AMP) still exists (27, 28). The amino acid sequences of CapB and PgsB share significant homology with Mur ligases of other bacteria, which take aminoacylphosphate.

AlbC

Streptomyces noursei and *S. alburus* have been known to produce an antibiotic compound, albonoursin, a dehydrated derivative of cyclo(Phe-Leu) (29, 30). A recombinant *S. lividans* expressing a gene from *S. noursei*, named *albC*, was found to synthesize cyclo(Phe-Leu) (31). The amino acid sequence of AlbC has no homology with any known NRPSs and dose not contain an ATP grasp motif, thus, the reaction mechanism of the enzyme remains unclear.

Application of peptide-bond forming activites in dipeptide production

(1) *Dipeptides' situation*

L-α-Dipeptides are the smallest peptides but versatile compounds. They have some characteristics which are absent in the corresponding amino acids. For instance, Gln is unstable in solution, but its dipeptide, Ala-Gln, is very stable and acts as Gln source once it was incorporated into a human body (32, 33). Tyr is hardly soluble but Ala-Tyr is 23-folds soluble. Certain kinds of dipeptides are also known to have specific physiological functions; Tyr-Arg has an analgesic effect (34), Val-Tyr has an antihypertensive effect (35), etc. Despite of these usefulness, commercial use of dipeptides is limited due to their unavailability at low cost.

There have been a variety of methods to synthesize dipeptides, which are categorized in two types; chemical synthesis and enzymatic synthesis. Chemical synthetic way, originated from Emil Fisher's work in 1901, generally requires protection and deprotection steps. Any kinds of dipeptide can be synthesized by chemical methods (36). However, because of the multi steps and the necessity of protection, their production costs are so high that they can not be applied in wider commercial use. Enzymatic method has also a lot of variations but commonly employs the reverse reaction of a protease or peptidase to connect amino acids (37, 38). To suppress the forward reaction, hydrolysis of the peptide bond, and to direct the order of the two amino acids, protection of amino- or carboxy-group of the amino acids is required as well as chemical synthetic method. Thus, application of enzymatic methods has also been limited. The ideal way to synthesize dipeptides is connecting unmodified amino acids in an irreversible reaction.

(2) *Emerging new technologies for dipeptide production*

Progress in the research on peptide-bond forming activities have been opened the possibility to synthesize a designed dipeptide from unprotected amino acids. Two lines of studies have been reported; NRPS utilizing method and Lal utilizing method.

NRPS method

The modular architecture of NRPS prompted to engineer the enzyme to synthesize a designed dipeptide.   Doeckel and Marahiel (39) designed synthetic templates for dipeptide formation by combining parts of *bac* (coding for bacitracin-biosynthetic NRPS in *Bacillus licheniformis*) and *tyc* (coding for tyrocidine-biosynthetic NRPS in *B. brevis*) genes. The artificial enzyme was expressed in *Escherichia coli,* purified, and incubated with Ile, Leu, and ATP. Ile-Leu was formed as expected. Using similar strategy, dimodular NRPSs which synthesized Asp-Phe were constructed (40). A-PCP or A-PCP-C domains of Asp-activating module from surfactin synthetase of *B. subtilis* and A-PCP, C-A, A-PCP, or A domains of Phe activating modules from tyrocidine synthetase were fused.   These artificial synthetases had Asp-Phe synthesizing activity, but their activities were significantly different, indicating the importance of the strategy to construct a hybrid NRPS.   Knowledge on NRPS and know-how for engineering it have been accumulating rapidly.   Theoretically, any dipeptide can be synthesized by a designed NRPS (41).   However, since C and Te domains have been revealed to have some preference on their substrates, careful design and some trial and error shall be needed to get a highly active enzyme.   These difficulties may cause the very low productivities in productions with whole cells.

Lal method

Considering the broad substrate specificity and simplicity of Lal, this enzyme was expected to realize a novel process, which would overcome the drawbacks of the current dipeptide manufacturing processes (chemical synthesis or protease/peptidase dependent methods).   Two types of the process have been invented; the resting cell reaction process and the direct fermentation process (Fig. 3).

The resting cell reaction process is a coupling reaction of Lal and an ATP-regeneration reaction.   Detergent treated *E. coli* cells expressing Lal from *B. subtilis* and polyphosphate kinase from *Rhodobacter sphaeroides* was reported to produce several kinds of dipeptides (Ala-Met, Ala-Val, Ala-Ile, Ala-Leu, Gly-Met, and Gly-Phe) by incubating the corresponding amino acids and polyphosphate (42). Ala-Met gave the highest titer, 127.9 mM (28 g/L), from 200 mM each of Ala and Met.   Any dipeptides within the product spectrum of Lal can be produced by changing the substrate amino acids.
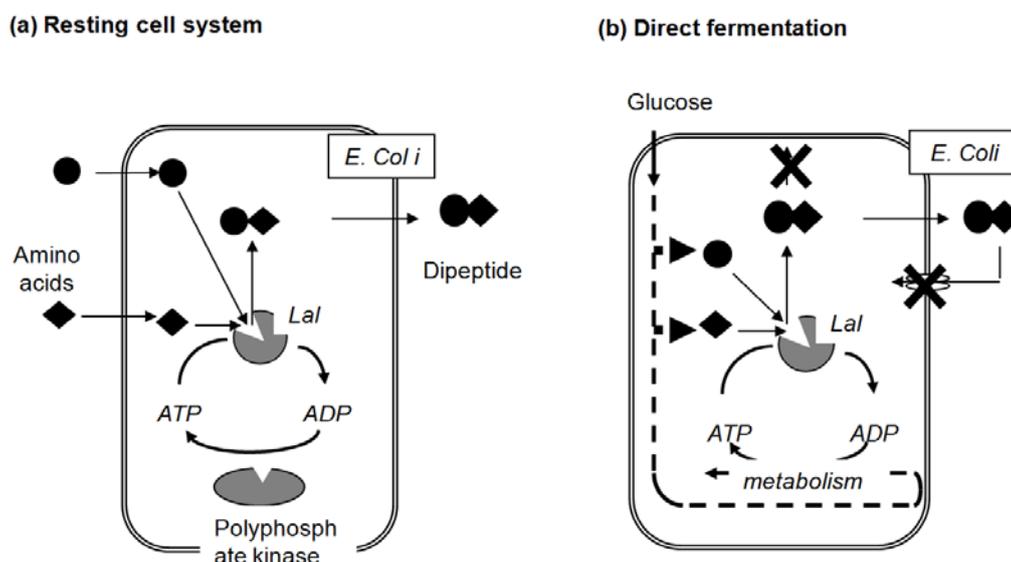

Fig. 3   Lal-based dipeptide production systems.
Diamonds and triangles indicate amino acids.

Because Lal takes unprotected usual amino acids as the substrates, Lal-expressing organism would produce some dipeptides without adding the amino acids. This conceptual idea of the direct fermentation was tested, but simply expressing Lal in *E. coli* resulted in no accumulation of dipeptides (43). Two obstacles were assumed; the relatively low affinity of Lal for amino acids and the dipeptide degrading activity of the host cells. To overcome these problems, enhancing the metabolic flux to the substrate amino acids and reducing the degradation activity, are necessary. Ala-Gln fermentation is a successful example (43). For the former purpose, Gln biosynthesis was deregulated and alanine dehydrogenase (Ald) from *B. subtilis* was co-expressed with Lal. Reduction of dipeptide degradation was achieved by the combinatorial disruption of genes for several dipeptidases (PepA, PepB, pepD, and PepN) and the dipeptide-import system (Dpp). Lal and Ald were expressed in the host strain under a stationary phase specific promoter to escape the harmful effect of Lal expression on cell growth. Fed-batch cultivation of the recombinant strain on a glucose-ammonium medium resulted in the accumulateion of Ala-Gln (100 mM) in the cultivation supernatant. No tripeptides or D-amino acid containing dipeptides were detected.

Some other producer strains for Ala-Met, and Thr-Phe, respectively, were also reported (44). Obviously, the direct fermentation method is the most cost-effective for dipeptide manufacturing since it dose not need even the substrate amino acids. However, there has been little insight into balancing two metabolic fluxes or the intracellular fate of dipeptides, those of which are important for the construction of the producer strain. The unexpected fact that Lal expression harmed cell growth should also be managed.

Perspectives

It is astonishing that nature prepares so many ways to form peptide bonds as described above. The activities are different in the flexibility, accuracy, reaction rate, and energy cost. The ribosome system is most flexible and highly accurate, but needs a huge and complex enzyme system. NRPS is also a huge enzyme and less flexible but can be designed to synthesize any peptide. Lal is a simple enzyme but lesser flexible. On the other hand, the high-energy phosphate bonds consumed to form one peptide bond are 4 (2 x ATP → AMP) in NRPS and 1 (ATP → ADP) in Lal. It would be interesting to know how organisms have appointed each activity for each job. While there are various kinds of peptide-bond forming activities, they can be divided into two types based on the reaction intermediate. Evolutional relation between the two types may be another interesting point. From the view point of application, nonribosomal peptide-bond forming activities should be useful for peptide production. Fermentative production of dipeptide using Lal is the pioneering example. Pursuing these possibilities will open the new filed of peptide application.

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