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Microbial production of natural poly amino acid

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Three kinds of poly amino acids, poly-γ-glutamic acid, poly(ε-*L*-lysine) and multi-*L*-arginyl-poly (*L*-aspartic acid) can be synthesized by enzymatic process independently from ribosomal protein biosynthesis pathways in microorganism. These biosynthesized polymers have attracted more and more attentions because of their unique properties and various applications. In this review, the current knowledge on the biosynthesis, biodegradations and applications of these three poly amino acids are summarized.

poly amino acid, biosynthesis, metabolism, application

1 Introduction

The term "polyamides" covers a large amount of polymer compounds with their constituents linked by amide bonds. The polyamides are divided into two categories: one is the poly amino acids, which are consisted by one type of monomers, and another is proteins, which are composed of different kinds of constituents. The overwhelming majority of polyamides are the co-polymeric proteins. A small group of polyamides are referred to as poly amino acids in order to distinguish them from the proteins due to different features of biosynthesis. There are several differences between them: (1) Protein is composed by variety of amino acid, whereas poly amino acid is consisted of only one type of amino acid, at least in the backbone. (2) Protein is biosynthesized under the direction of DNA, which means the protein synthesis is in a template-dependent mode, including complex ribosome transcription and translation mechanism. Because of the biosynthesis of poly amino acid is catalyzed by some simple enzymes, therefore, the inhibitors of translation such as chloramphenicol do not affect the biosynthesis of poly amino acids. The enzymes catalyzing the biosynthesis of poly amino acid are grouped into the carbon-amino binding enzymes. (3) The proteins exhibit exact length, whereas poly amino acids show remarkable dispersal in molecular weight. (4) While amide linkages in proteins are only formed between α-amino and γ-carboxylic groups(α-amide linkages), amide bonds in poly amino acids involve other side chain functions(i.e. β- and γ-carboxylic and ε-amino groups).

There are three different poly amino acids presented in the nature: poly-γ-glutamic acid (γ-PGA), poly-ε- lysine(ε-PL) and cyanophycin. The chemical structures of these three kinds of poly amino acids are shown in Figure 1. γ-PGA is made of *D*- and *L*-glutamic acid units linked by amide linkages between α-amino and γ- carboxylic acid groups[1]. ε-PL is consisted of lysine monomer by the linkage of α-carboxyl group and ε-amino group of lysine[2]. Different from these two kinds of poly amino acids, the constituents of the third poly amino acid, cyanophycin, are α-aspartic residues containing pendent arginine residues linked to the β-carboxyl group[3].

In this review we present a comparative survey of the structure, the biosynthesis, the biodegradation and the technical applications of the naturally occurring poly

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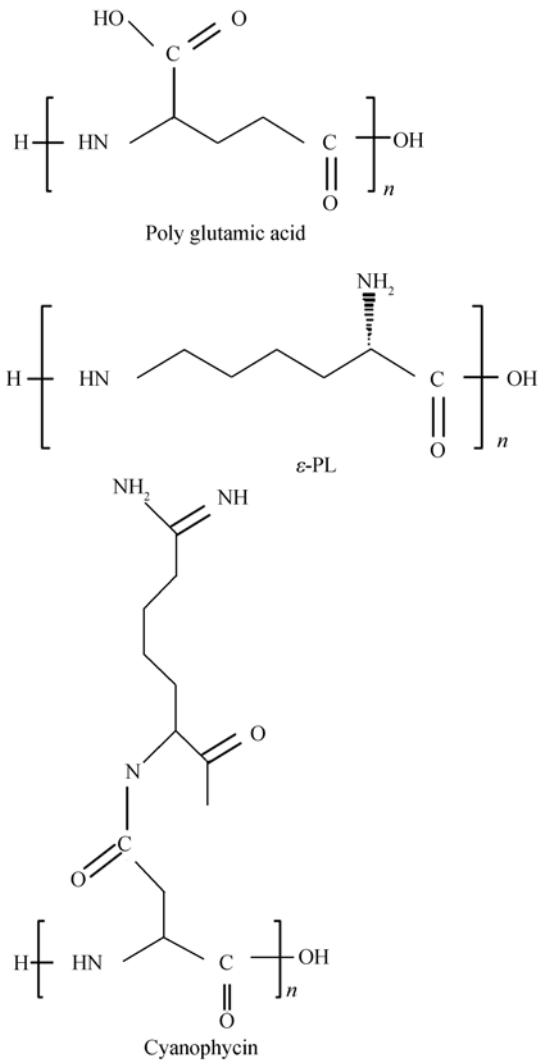

Figure 1 The chemical structure of three poly amino acid.

amino acids with particular emphasis on recent insights into the biochemistry and molecular genetics of these highly interesting compounds.

2 Poly-γ-glutamic acid (γ-PGA)

γ-PGA, which is a kind of water-soluble and biodegradable polymer, is made of *D*- and *L*-glutamic acid units linked by amide linkages between α-amino and γ-carboxylic acid groups. Multifarious applications have been developed in the food and cosmetic as well as pharmaceutical industries. High water-solubility and biodegradability of γ-PGA and its derivatives make them potential substitutes for hydro gels and thermoplastic polymers, traditionally synthesized from petroleum[4]. Furthermore, γ-PGA may function as an adaptation agent in various environmental applications, such as heavy metal ion absorbent[5].

2.1 Microorganisms and metabolic pathway for γ-PGA production

2.1.1 γ-PGA producing microorganisms. γ-PGA was first discovered by Ivanovics as the capsule of gram-positive bacterium *Bacillus anthracis*[6]. Then γ-PGA was also discovered in other *Bacillus* sp., such as *licheniformis*, *B. megaterium*, *B. subtilis*, and *B. amyloliquefaciens*[7−11]. It is well known that the mucilage of "natto" is a mixture of γ-PGA and fructan produced by *Bacillus natto*[12]. The major γ-PGA producing microbes are shown in Table 1. Except *Bacillus* sp. in the nature, two halophilic eubacteria, *Sporosarcina halophila*, *Planococcus halophila*[13] and a halophilic archaebacterium, *Natrialba aegyptiaca* sp.[14, 15] has also been found to have the ability of excreting γ-PGA. In addition to prokaryotes, γ-PGA is detected in significant amount in the nematocysts of *Cnidaria*[16].

2.1.2 Biosynthesis and metabolic pathways of γ-PGA. Many efforts have been paid to clarify the enzymes related to the biosynthesis of γ-PGA in order to increase its productivity. The metabolic pathways for γ-PGA synthesis are becoming known. The results indicate that the key intermediate of γ-PGA synthesis is 2-oxoglutarate, which is a direct *L*-glutamic acid precursor[17,18] and is synthesized via glycolysis and tricarboxylic acid cycle. It is found that in *B. licheniformis* two enzymes related to *L*-glutamic acid, namely glutamate synthase(EC 1.4.1.13) and glutamate dehydrogenase(EC 1.4.1.3) are insensitive to the end-product concentration, which re-

Table 1 The *Bacillus* sp. γ-PGA producers ZJU-7 to other strains reported before

Strain	Main nutrients(g/L)	Culture time(h)	γ-PGA(g/L)	Molecular weight(Dolton)
B. licheniformis ATCC 9945a	Glutamine(20), glycerol(80), citric acid(12)	96	17−23	$1.4-9.8\times10^5$
B. subtilis IFO3335	Glutamine(30), citric acid(20)	48	10−20	$1.0\times10^5-2.0\times10^6$
B. subtilis TAM-4	Fructose(75), NH_4Cl(18)	96	22	$6.0\times10^5-1.6\times10^6$
B. subtilis F-2-01	Glutamine(70), glucose(1)	96	48	1.2×10^6
B. subtilis ZJU-7	Sucrose(60), Glutamine(80)	24	54.4	1.23×10^6

sults in high intracellular concentration of *L*-glutamic acid and is favorable to increase the productivity of γ-PGA[19].

Based on the research works of the biosynthesis pathway of γ-PGA in *B. anthracis*, a membrane bound γ-PGA synthetase system is identified[20]. This enzyme system contains at least three enzymes and a sequence-reaction mechanism is suggested as followings:

L-Glutamic acid+ATP→γ-*L*-glutamyl-AMP+Ppi (1)

γ-*L*-glutamyl-AMP+SH-enzyme→γ-X-glutamyl-S-enzyme+AMP (2)

γ-X-glutamyl-S-enzyme→γ-*D*-glutamyl-*S*-enzyme (3)

γ-*D*-glutamyl-S-enzyme+[γ-*D*-glutamyl]$_n$→[γ-*D*-glutamyl]$_n$+1+SH-enzyme (4)

It is clear that *L*-glutamic acid is activated by phosphoration in the first step, which means that energy must be supplied for the biosynthesis of the amide bond of γ-PGA. The isomerization reactions of *L*-glutamic acid in *B. anthracis*, described as reaction (2) or (3), are still under questioning, because in *B. subtilis* IFO3335, the results show that *L*- as well as *D*-glutamic acid can be incorporated into the polymer without different[21].

The synthesis of γ-PGA is a ribosome independent, but an enzyme catalyzed process. In addition, γ-PGA is an extracellular product. The identification of genes and the determination of the location of enzyme, related to the biosynthesis of γ-PGA, are important to understand the biosynthesis and secretion of γ-PGA. It has been reported that the genes related to γ-PGA synthesis in *B. anthracis* lie on the plasmid DNA[22]. However, in *B. subtilis* TAM-4, there was no plasmid and the genes responsible for γ-PGA lie on genome DNA.

Recently, Ashiuchi screened the genes encoding γ-PGA biosynthesis enzymes in *B. subtilis* (*natto*)[21]. A membrane bound γ-PGA synthetase system can explain the mechanism of γ-PGA how the amide bond to be synthesized, chain to be extended and γ-PGA to be secreted into the environment. Also, Ashiuchi and his co-workers screened *E. coli* clones consisting the genome DNA of *B. subtilis* IFO3336. One positive clone having the ability of γ-PGA synthesis was screened in about ten thousands clones. Sequencing of the gene containing in the clone shows the gene containing three ORFs and named as *pgs*B, *pgs*C and *pgs*A. To know if the system occurs ubiquitously in γ-PGA producers, long-PCR was performed on the chromosomal DNA of *B. subtilis* IFO 3013, IFO 3335 and IFO 13169, *B. subtilis* (*natto*) A, B, C, and D isolated from commercially available *natto* foods, as well as *B. licheniformis* IFO 12107 (ATCC9945a) and *B. subtilis* (*chungkookjang*). The DNA fragments (about 3.0 kb) are found in the PCR products of all the strains tested. To further determine whether a γ-PGA synthetic system other than the *pgs*B, C and A system operate in *B. subtilis*, the *pgs*B, C and A gene-disrupted of *B. subtilis* (*chungkookjang*) has been constructed and the γ-PGA productivity of the mutant examined. The results show that all mutants lose the polymer synthesis ability. They concluded that genes *pgs*B, C and A are exclusively responsible for γ-PGA biosynthesis. Based on the results above, the function of *pgs*B, *pgs*C and *pgs*A are also studied. The *pgs*A may function as a γ-PGA transporter. It seems likely that this function of *pgs*A is important for the extension of γ-PGA chain and secretion. A structural feature commonly seen in amide ligases is found in *pgs*B and the consensus sequence of the ATP-binding motif lies on the residues 37±42 (GIRGKS) of the protein. The *pgs*C may be essential in γ-PGA synthesis, however, the function of this gene is still unknown[23].

2.1.3 Biodegradation of γ-PGA. One of the unique features of γ-PGA is the biodegradability. All γ-PGA producing strain can also metabolize γ-PGA as carbon resource as well as nitrogen resources. A depolymerase has been identified in the fermentation broth of *B. licheniformis 9945a*. The enzyme is physically bound to the γ-PGA around the cell and responsible for γ-PGA degradation[24].

The ability of other natural organisms for γ-PGA degradation is also examined. A medium containing γ-PGA as the only carbon and nitrogen resource was designed to test various microbes. At least twelve strains having the ability to degrade γ-PGA were isolated[25]. Besides biodegradation, although aqueous solutions of γ-PGA is rather stable at room temperature, the amide will be broken when the temperature risen up to 60℃[26].

2.2 High productive strains, medium design and fermentation processes for γ-PGA production

The γ-PGA producing strains are divided into two categories: *L*-glutamine acid-dependent and *L*-glutamine acid-independent strains. Although it seems better to choose *L*-glutamine acid-independent strains in industry

use, little is known about these bacteria. Regardless research works and industrial applications, the production of γ-PGA is focused on the *L*-glutamine acid-dependent strains. The strain characteristics, medium design and fermentation processes will be discussed in detail for major high productive strains.

2.2.1 γ-PGA production by *B. licheniformis 9945a*. *B. licheniformis 9945a* was found to be a γ-PGA producer in 1942[7]. Since then, systematic study on the medium design and fermentation conditions has been carried out[27−30]. It is found that the concentrations of salt, *L*-glutamic acid, glycerol and citric acid in the medium are most important factors affecting the productivity of γ-PGA. Mn^{2+} and Ca^{2+} have significant influent on γ-PGA production[31]. The optimized medium compositions for production are: *L*-glutamic acid 20 g/L, citric acid 12 g/L, glycerol 80 g/L, NH_4Cl 7 g/L, $MgSO_4$ 0.5 g/L, $FeCl_3$ 0.04 g/L, K_2HPO_4 0.5 g/L. The pH value of the culture medium should be adjusted to 7.4 with NaOH. After 2−3 d cultivation, the γ-PGA productivity of 15 g/L can be reached.

There is a long-standing problem in the producing of γ-PGA by *B. licheniformis 9945a*: the strain readily degenerate to a non-γ-PGA-producing strain after repeated cultivation. This instability caused extreme diverse results from culture to culture in both γ-PGA productivity and fermentation kinetics[8,27,32]. This problem is finally solved by Birrer[29] using cryogenically frozen vegetative cells, by which, consistent results were observed in batch culture using complete or modified medium E.

2.2.2 γ-PGA production by *B.subtilis* IFO3335. *B. subtilis* IFO3335 is isolated in a traditional Japanese food named "*natto*". This bacterium is also intensively studied for the production of γ-PGA with the productivity ranging from 20 to 50 g/L under various culture conditions[33,34]. The typical medium composition is: 30 g/L *L*-glutamic acid, 20 g/L citric acid and 5 g/L ammonium sulfate and the cultivation period is about 96 h.

It is observed that without addition of *L*-glutamic acid in the medium, no γ-PGA is produced, whereas, if *L*-glutamic acid is added into the medium, its concentration in the broth remains almost unchanged although γ-PGA is synthesized in large amount. It seems that *L*-glutamic acid added in the medium is merely acted as an activator of enzymes responsible for the biosynthesis of γ-PGA. The *L*-glutamic acid, as the precursor of γ-PGA synthesis, is the metabolite of citric acid and ammonium sulfate by cell itself. When citric acid is used as sole carbon source, γ-PGA is the only product. If other carbon sources, such as glucose, sucrose and acetic acid, are used, polysaccharide will be the predominant product in the broth with little γ-PGA produced. Moreover, the productivity of polysaccharides will decrease with the increase of ammonium sulfate concentration in the medium. As the ammonium sulfate concentration reached 5 g/L, γ-PGA becomes the dominant product in the broth again.

2.2.3 Production by *B. subtilis* ZJU-7. A novel strain with high γ-PGA productivity has been isolated from fermented bean curd, a traditional Chinese food in this lab[35]. The strain is named *Bacillus subtilis* ZJU-7 according to 16s RNA sequencing and its taxonomic characters. The most suitable carbon and nitrogen sources are sucrose and tryptone respectively. Exogenous *L*-glutamic acid is necessary for γ-PGA production and the productivity of γ-PGA increased upon the addition of *L*-glutamic acid to the medium. In the culture medium containing 60 g/L sucrose, 60 g/L tryptone and 80 g/L *L*-glutamic acid and after cultivated at 37℃ for 24 h, the yield of γ-PGA reached 54.4 g/L, which is the highest γ-PGA productivity ever reported. The average molecular weight is about 1.24×10^6 Dolton.

2.2.4 γ-PGA production by co-cultivation. Most industrial important γ-PGA producing strains are *L*-glutamic acid dependent. In order to avoid exogenous addition of *L*-glutamic acid and decrease the cost of γ-PGA producing, a new research work in this laboratory is making progress. A co-cultivation system consisted of a *L*-glutamic acid producer *Corynebacterium glutamic- um* S9114 and a γ-PGA producer *Bacillus Subtilis* ZJU-7 has been established (not published data). In this system, glucose is the sole carbon source. *Bacillus Subtilis* ZJU-7 will consume the *L*-glutamic acid produced by Corynebacterium glutamicum S9114 for γ-PGA biosynthesis. It is found that the main factors influencing the co-cultivation system are: the seed age of both strains, the ratio of the two strains at the beginning of co-cultiv- ation and the urea concentration in the culture medium.

At first, *Corynebacterium glutamicum* S9114 and *Bacillus subtilis* ZJU-7(in *L*-glutamic acid-free medium) are cultivated separately, then *Bacillus subtilis* ZJU-7 is added into the fermentation broth of *Corynebacterium*

glutamicum S9114 to reach a ratio of 56∶1. The co- cultivation is performed at 37℃ and urea is added to the co-cultivation system to keep the pH at 7. The time courses of co-cultivation are shown in Figure 2. The distinquished feature of the co-cultivation is that the ratio of *Corynebacterium glutamicum* S9114 and *Bacillus subtilis* ZJU-7 is lowered down from 56∶1 to 2.4∶1, whereas the total cell density keeps increasing. The concentration of *L*-glutamic acid increases initial, then passes a maximum and decreasing because of the consumption for γ-PGA synthesis. The highest γ-PGA concentration of 32.8 g/L was observed after fermentation for 24 h.

Except for the strains introduced above, several *L*-glutamic acid-independent strains also produce γ-PGA, such as *B. licheniformis* A35 and *B. licheniformis* S173[36,37].

2.3 Separation, purification and identification of γ-PGA

γ-PGA can be purified by the methanol precipitation method[26] or copper sulfate method[32]. The molecular weight can be determined by GPC[38] method or SDS-PAGE and stained with Alcian blue or other basic dyes[39]. The range of molecular weight of γ-PGA is from 1.0×10^5 to 1.0×10^6 dalton. The monomer can be determined by HPLC after complete hydrolyzation of γ-PGA. The conformation of γ-PGA in solution has been extensively investigated using various methods and the results show the structures are different according to the ionization degree of γ-PGA[40]. The unionized polymer is reported to take a helical conformation, while the ionized polymer is in a random coil state.

2.4 Application

As a natural biopolymer, γ-PGA has many applications in different field. It can be used as a drug carrier in medicine to increase drugs solubility and decrease their side-effect[41]. It also can be used as biological adhesives in sealing air leakage from the lungs[42]. Because of the high affinity with water and heavy metal ion, γ-PGA can be used in cosmetic as a moisture container[43] and as an absorbing material in wastewater treatment[44].

3 Cyanophycin

3.1 Characteristics of cyanobacteria

Cyanophycin, which is also referred to as cyanophycin granule polypeptide(CGP), was first discovered more than 100 years ago[45]. The primary structure of cyanophycin is shown in Figure 1. The strand of the polymer consists of almost equivocal amounts of arginine and aspartic acid arranged as a poly aspartate backbone, with arginine moieties linked to the β-carboxyl group of almost every aspartic acid residue; the polyamide is highly dispersal, exhibiting a molecular weight ranging from 2.5×10^4 to 1.0×10^5 dalton as estimated from SDS- PAGE

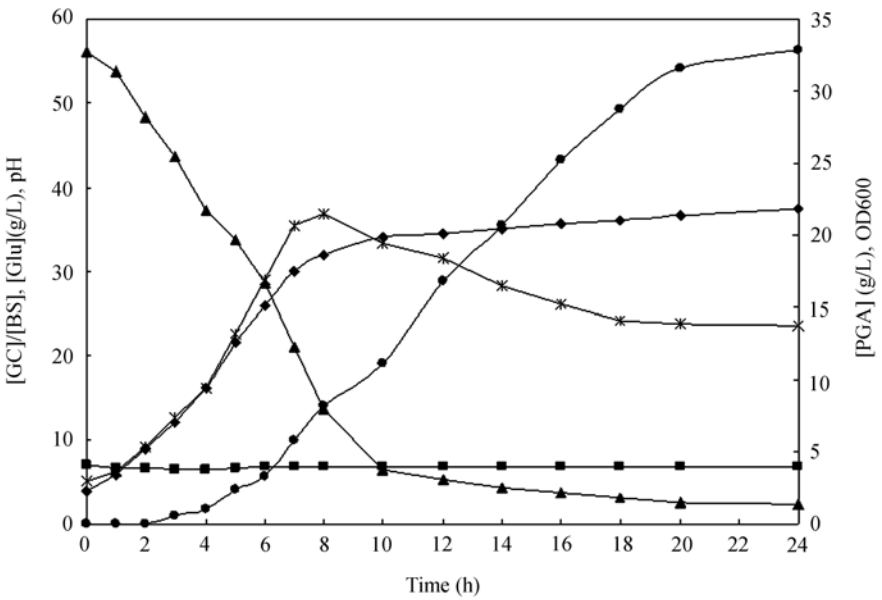

Figure 2 The time course of co-cultivation system under the optimized culture conditions. ◆, Cell optical density, OD_{660}; ●, γ -PGA concentration; ▲, the ratio between *Corynebacterium glutamicum* and *Bacillus subtilis*; *, *L*-glutamic acid concentration; ■, pH of the culture medium.

corresponding to polymerization degrees of 90 to 360. Cyanophycin is not affected by a variety of proteases such as pronase, pepsin and chymotrypsin.

In contrast to γ-PGA and ε-PL, cyanophycin is located only inside the producing cells. Cyanophycin seems to be a compound whose distribution is limited to cyanobacteria[46]. As no ultra-structural evidence exists so far for the occurrence of similar inclusions in other organisms, and as it is not possible to identify material with the typical characteristics of cyanophycin from other sources. The granules made of cyanophycin vary in size and shape[47,48] and occur in all cyanobacterial groups: unicellular and filamentous, nitrogen-fixing and non-nitrogen-fixing[49−52]. Cyanobacteria can be divided into two categories: heterocyst-forming cyanobacteria and non-heterocyst-forming cyanobacteria. In non- heterocyst-forming cyanobacteria, the cyanophycin granules are distributed in the protoplasts[53]. In heterocyst-forming cyanobacteria, cyanophycin granules are frequently present in the heterocysts, which are specialized for the fixation of molecular nitrogen. In those cells the cyanophycin granules are primarily associated with the polar nodule and the neck, which connects the heterocyst to the adjoining vegetative cell[54].

3.2 Culture conditions and metabolic pathway of cyanophycin

3.2.1 Culture conditions for cyanophycin biosynthesis. Cyanophycin is used as a temporary nitrogen reserve in the cells: during the transition of the exponential to the stationary growth phase it accumulates and disappears when balanced growth resumes[55]. In the N_2-fixing heterocysts, cyanophycin appears in the late stages of heterocyst maturation[54]. Therefore, the cyanophycin content is low in exponentially growing cells, but increases during stationary phase to amounts of 8%−18% (*w*/*w*) of the cell dry mass[51,54].

From the microorganism's point of view, poly arginine should be a better intracellular nitrogen reserve than cyanophycin. Cyanophycin containing five nitrogen atoms for every building block of the polymer, and it is insoluble at internal pH and ionic strength in the cells. Whereas poly arginine contains more nitrogen according to unit mass, and is soluble under physiological conditions. However, poly arginine would therefore have severe effects on the cell internal milieu due to binding to poly anionic DNA and changing in the osmolarity inside the cell[47]. When the cyanobacteria are cultured in medium containing low ammonium concentration, it begins to synthesis cyanophycin before ammonium being exhausted. The cells thus appear to "sense" a declining concentration of extracellular nitrogen and trigger cyanophycin synthesis when the ammonia concentration reaches a low level before it is completely exhausted. Such "forward planning" gives cyanobacteria a competitive advantage over other organisms[54].

Based on the characters of cyanobacteria discussed above, several strategies have been reported to enhance the synthesis and accumulation of cyanophycin, such as deprivation of sulfur[56,57] or phosphorus[51,58] and the addition of translatable or transcriptional inhibitors[50,59−61]. The accumulation of this polymer increases with the addition of a variety of nitrogen-containing compounds to the medium, such as ammonium, urea, glycine, aspartic acid, and arginine[62,63]. Moreover, cyanophycin content also rise when cultivation was under reduced light and temperature[64].

3.2.2 Biosynthesis pathways of cyanophycin. Cyanophycin synthetase is the only enzyme responsible to the biosynthesis of cyanophycin. Ziegler et al. reported that *A. variabilis* completely lost its cyanophycin biosynthesis ability after knocking out of cyanophycin synthetase encoding gene *cphA*[65]. The *cphA* gene has been cloned and sequenced from several cyanobacteria. In all cases the cyanophycin synthetase encoding gene *cphA* is located in co-linear orientation close to and downstream from the gene *cphB*, which encodes the intracellular cyanophycinase, thus forming the *cph* gene cluster. Sequence analysis of the translation product of cyanophycin synthetase encoding gene in different strains shows that these enzymes are highly similar in preliminary structure and each of them has ATP-dependent carboxylate-amine/thiol ligases in the N-terminal/central region and ATP-dependent substrate ligases in the central/C-terminal region**.**

First characterization of cyanophycin synthetase is done by Simon[66]. The enzyme being enriched 92-fold is extracted from the soluble cell fraction of *Anabaena cylindrica*. The polymerization reaction catalyzed by the enzyme in vitro dependents on the presence of both monomers, ATP, Mg^{2+}, K^+, a sulphydryl reagent and small amounts of cyanophycin as primer. Cyanophycin synthetase derived from other strains has the same dependencies, such as *A. variabilis*[67], thermophilic *Synechococcus* sp. strain MA19[68], and *Synechocystis* sp.

strain PCC 6308[69]. Cyanophycin synthetases from these sources consist of identical subunits of 90—130 kDa, respectively, which most probably assembles two homodimers under physiological conditions.

It is also been proved that the synthetase can only prolong preexisting cyanophycin primers with the presence of both amino acid substrates. Absence of any substrate would result in no prolong incorporation of the other. With the improvement of enzyme activity measurement technique, the K_m value has also been tested. The K_m value of *L*-arginine, *L*-aspartic acid and ATP are 49, 0.45 and 0.20 μM respectively. This means high affinity of the enzyme to these substrates.

3.2.3 Biodegradation of cyanophycin. Cyanophycin can be depolymerized by intracellular cyanophycinase, which also has been isolated from *Synechocystis* sp. strain PCC6308[70]. However, the final product isn't amino acid monomer but dipeptide consisting of *L*-arginine and *L*-aspartic acid. The enzyme that decomposes dipeptide to single monomer is still unknown. It is assumed that *L*-arginine and *L*-aspartic acid may be catabolized in a special *L*-arginine catabolic routine, which can make the fixed nitrogen available to the cell. The decomposing of cyanophycin is still not reported in the literature. However, the microorganism that can use cyanophycin as the only carbon and energy source has been isolated recently[71,72].

3.3 Separation, purification and identification of cyanophycin

Cyanophycin granules can be purified by differential centrifugation: after cell disruption centrifugation is performed for 10 min at 20000 *g*. Then the orange- colored upper part of the pellet was resuspended in distilled water and centrifuged again for 30 s at 1000 *g*; centrifugation of the supernatant at 1000 *g* for another 15 min gave a pellet whose lower white layer consisted almost entirely cyanophycin granules. Further more, the polypeptide is insoluble in organic solvents, cyanophycin also can be purified by organic solvent precipitating method[66].

Circular Dichroism(CD) and Laser Raman Spectroscopy results show that cyanophycin exhibited a defined secondary structure(β-sheets) in acidic solutions but not under alkaline conditions. Standard method for quantifying the amount of cyanophycin in cells is HPLC[73]. However, a more rapid and sensitive method for the quantification of the polymer from crude preparations on the basis of ^{1}H NMR has been described[74].

3.4 Application

As a macro molecular, cyanophycin has various advantages: high solubility, high viscosity and biodegradability. The applications of cyanophycin are similar with that of γ-PGA including medicine, cosmetics, environment protection and agriculture[75]. However, because of much higher production cost of cyanophycin, it is hard to use it in large scale. Recently researchers try to synthesis cyanophycin in predominated *E. coli* containing *cphA* gene to reduce the cost[69]. The highest productivity is about 26% of all dry cell weight after cultivation in very rich, complex and cost medium; otherwise, the cell density and cyanophycin productivity are quite low. The low cell density is probably caused by the exhaust of intracellular amino acids, such as *L*-arginine, *L*-aspartic acid and glutamine, the precursor of *L*-arginine. New efforts are in progress to reduce the production cost of cyanophycin by cloning *cphA* gene into, many other industry bacteria, such as *Corynebacterium glutamicum*, *Ralston eutropha* and *Pseudomonas* spp[76—78].

4 Poly(ε-*L*-lysine)

Poly (ε-*L*-lysine) (ε-PL) is a homo-poly-amino acid characterized by the peptide bond between the carboxyl and ε-amino groups of *L*-lysine. ε-PL was first discovered by Shima and Sakai in 1977[79]. They found a Dragendroff-positive compound in the culture medium of a strain named *Streptomyces albulus* 346 and proved the compound was a homo-poly-amino acid consisting of *L*-lysine. The length of ε-PL produced by the strain is about 25 *L*-lysine monomers[79,80]. ε-PL with different length has also been discovered in several strains(length 10—36, 10—24, 8—9)[81,82].

4.1 Microorganisms and metabolic pathways of ε-PL

4.1.1 Biosynthesis of ε-PL by *Streptomyces albulus*. To isolate ε-PL producing strains, an acidic dye, Poly-R-487 was used. The acidic dye was added to the screening agar medium and the microorganisms were coated on the plate. When the strains produced basic extracellular polymers such as ε-PL, Poly-R-487 was condensed around those colonies. By this method, several strains were screened, such as *Streptomyces, Kita-*

satospora, *Epichloe* and an ergot fungus, *Epichloe* sp. MN-9[81].

Various ε-PL producers have been reported in the literatures. In Japan, ε-PL has been commercialized by a mutant strain derived from *Streptomyces albulus* 346[79]. At the beginning of fermentation of wild type *Streptomyces albulus* 346, the maximum productivity of ε-PL is only about 0.5 g/L under the optimized culture condition. The pH control of broth was essential for the accumulation of ε-PL during the fermentation process. The initial pH value of culture medium is kept at 6.0 and when the cells grow to stationary phase, ε-PL accumulation could be observed in broth. To produce ε-PL efficiently, the pH value of broth must be regulated in the range of pH 3.0 to 5.0. They found that the addition of *L*-lysine as precursor to the fermentation broth did not increase ε-PL productivity. Subsequently, the ε-PL productivity by resting cells using glucose and $(NH_4)_2SO_4$ as substrates were also tested. Under acidic pH conditions, ε-PL accumulation was improved greatly and a maximum productivity of 4—5 g/L was obtained.

To increase ε-PL productivity, the mechanism and metabolic pathway of ε-PL biosynthesis were studied extensively. The results show that *L*-lysine caused partial repression of the synthesis of aspartokinase, a key enzyme for *L*-lysine biosynthesis, and glycine inhibited aspartokinase activity. Based on above mechanism, a *S*-(2-aminoethyl)-*L*-cysteine, an analogue of *L*-lysine, plus glycine-resistant mutants were derived from *S. albulus* 346. The growth of original strain is depressed by 1 g/L glycine, but the mutant is able to grow in a medium containing 3 g/L of *S*-(2-aminoethyl)-*L*-cysteine without depression effect. By this method, more than 90% of *S*-(2-aminoethyl)-*L*-cysteine and glycine- resistant mutants isolated are high ε-PL productivity strains. *S. albulus* 11011A, a strain with the highest ε-PL productivity, excretes 2.11 g/L of ε-PL in the broth under tube-cultivation. In 3 L fermentor, the ε-PL productivity of this strain reaches 20 g/L, with glucose and ammonium sulfate feeding and under continuous pH control after 120 h fermentation[83].

S. albulus 410(S410), one of the ε-PL producers exhibiting high productivity has been used to evaluate the effect of pH value on ε-PL productivity[84]. During the fermentation, the pH of the culture broth gradually decreased from its initial value of pH 6.8 to pH 4.2 by 36 h, and then further to pH 3.2 at 96 h. After the pH was lower than 4.2, ε-PL started to accumulate in the broth. They found that the fermentation process was divided into two phases: first phase for cell growth at relative high pH value of pH 5.0 and the second phase for ε-PL accumulation at low pH value of pH 4,0. The concentration of glucose in the broth is controlled at 10 g/L because the depletion of glucose would lead to a pH increase of the broth resulting in the degradation of the produced ε-PL. In this pH-control fermentation strategy, ε-PL productivity in a batch culture was enhanced from 5.7 to 48.3 g/L.

4.1.2 The biosynthesis genes and enzymology of ε-PL biosynthesis. The exact nature of ε-PL producing enzyme is still unknown. However, it has been reported that there is some relationship between ε-PL-producing enzyme and ε-PL-degrading enzyme[85,86]. Some ε-PL producing strains with high productivity, such as *S. virginiae* IFO12827 and *S. noursei* IFO15452 showed significant ε-PL-degrading aminopeptidase activity. As ε-PL has antibiotic activity, the ε-PL-degrading enzyme may play a protection role for ε-PL producers to tolerate the ε-PL secreted by the cell. This correlation suggests that ε-PL producers commonly possess the membrane-bound ε-PL-degrading enzyme. It has found that the ε-PL-degrading enzyme of *S. albulus* is tightly bound to the cell membrane and classified as a kind of aminopeptidase containing Zn^{2+}. The enzyme is purified to homogeneity, with a subunit molecular mass of 54 kDa. The most suitable pH value of the enzymatic hydrolyzation is pH 7.0, which can reasonably explain why low pH during ε-PL accumulation in the broth should be kept.

To clarify the ε-PL biosynthesis mechanism, it is very important to identify and analyze the relevant genes in *S. albulus*. There are several reports on the genes analysis of *S. albulus*, but unfortunately, the genes necessary for ε-PL production still have not been identified and analyzed. It has been reported that a high molecular weight plasmid, pNO33, might be involved in the ε-PL biosynthesis in *S. albulus*[87]. The mutant strains of *S. albulus* without this plasmid lose ε-PL producing ability. The plasmid has been isolated and the sequence has been partially determined. However, the function of this plasmid kept unknown and the complete sequence hasn't been reported yet.

As discussed above, there is a close relationship be-

tween ε-PL producing enzyme and ε-PL degrading enzyme. ε-PL degrading enzyme has been isolated and cloned from *S. albulus*[88]. The deduced amino acid sequence from the gene encoding ε-PL degrading enzyme shows 70% identity with a putative metallopeptidase isolated from *S. coelicolor*. The flanking regions of the gene for ε-PL-degrading enzyme have not been located yet. It is unknown whether the gene(s) for the biosynthesis of ε-PL are localized in the region adjacent to the gene for the ε-PL-degrading enzyme.

4.1.3 Biodegradation of ε-PL. ε-PL is rather stable in aqueous solutions. After boiled at 100℃ for 30 min or autoclaved at 120℃ for 20 min, no degradation is found and the polymer length remains unchanged[89]. However, there are many bacteria that can secrete ε-PL degrading enzyme to decompose the polymer. These bacteria are divided into two categories: one is the ε-PL producer and another is non- ε-PL-producer but can tolerate ε-PL antibiotic activity. Many ε-PL tolerant bacteria, such as *Sphingobacterium multivorum* and OJ10 and *Chryseobacterium* sp. OJ7, were isolated in the ε-PL rich medium. Both bacteria can grow well in presence of 10 g/L of ε-PL. The ε-PL degrading enzyme produced by *S. multivorum* OJ10 has been isolated and purified by Takimoto et al.[90]. This enzyme can be activated by Co^{2+} and Ca^{2+} and is able to decompose many peptides. The mode of ε-PL degradation is exotype and the enzyme releases N-terminal *L*-lysines one by one, and either *L*-lysyl-or *L*-arginyl- and *L*-leucyl-*p*-nitroanilides are efficiently hydrolysable by the enzyme.

Some bacteria could excrete ε-PL degrading enzyme and degrade ε-PL endo-type. The enzyme is isolated from *Chryseobacterium* sp. OJ7. It is the ε-PL degrading enzyme that enables the strain growth in the presence of high concentration of ε-PL. Moreover, some commercially available proteases, such as protease A, protease R and protease P, also have the ability of degrading ε-PL endo-type[90].

4.2 Separation, purification and identification of ε-PL

ε-PL is a cationic polymer in neutral pH. Due to this character, the polymer can be separated and purified by

Table 2 Minimum inhibitory concentration for the growth of microorganisms

Group	Species	MIC[a](μg/mL)	Medium[b]
Fungi	*Aspergillus niger* IFO4416	250	1
	Trichophyton mentagrophytes IFO7522	60	1
Yeasts	*Candida acutus* IFO1912	6	2
	Phaffia rhodozyma IFO10129	12	2
	Pichia anomala IFO0146	150	2
	P. membranaefaciens IFO0577	<3	2
	Rhodotorula lactase IFO1423	25	1
	Sporobolomyces roseus IFO1037	<3	2
	Saccharomyces cerevisiae	50	2
	Zygosaccharomyces rouxii IFO11301	50	1
Gram-positive bacteria	*Geobacillus stearothermophilus* IFO12550	5	6
	Bacillus coagulans IFO12583	10	6
	B. subtilis IAM1069	<3	7
	Clostridium acetobutylicum IFO13948	32	8
	Leuconostoc mesenteroides IFO3832	50	3
	Lactobacillus brevis IFO3960	10	3
	L. plantarum IFO12519	5	3
	Micrococcus luteus IFO12708	16	6
	Staphylococcus aureus IFO13276	12	4
	Streptococcus lactis IFO12546	100	3
Gram-negative bacteria	*Raoultella planticola* IFO3317	8	4
	Campylobacter jejuni	100	9
	Escherichia coli IFO13500	50	4
	Pseudomonas aeruginosa IFO3923	3	4
	Salmonella typhymurium	16	4

a) MIC: Minimum inhibitory concentration. b) Medium composition: 1, potato dextrose broth(pH 5.6); 2, malt broth(pH 5.0); 3, glucose malt broth(pH 6.0); 4, nutrient broth(pH 7.0); 5, deoxycholate broth(pH 7.4); 6, brain heart infusion broth(pH 7.0); 7, trypto-soya broth(pH 7.3); 8, thioglycollate medium without indicator(pH 7.1); 9, brucella broth.

the method of ion exchange chromatography. Then by further treatment of active charcoal and precipitating by organic solvent such as methanol or ethanol, purified ε-PL can be obtained.

4.3 Application of ε-PL

ε-PL is of antibiotic activity and the mainly used for food industry. Its antibacterial spectrum is shown in Table 2. It is obvious that of ε-PL is safe and the Minimum inhibitory concentration (MIC) value is rather low. Due to its low MIC concentration, the concentration of ε-PL necessary for food preservation is very low without causing bitter taste. Moreover, by combining ε-PL with other food additives, such as glycine, vinegar, ethanol and thiamine laurylsulfonate, the efficiency for various foods preservation will be enhanced. The safety of ε-PL in food industry has been proved by experiment using rat[91]. From various evaluation strategies, ε-PL is proven a safe food additive and causes no toxicity in reproduction, neurological and immunological functions, embryonic and fetal development, and growth of offspring and development of embryos or fetuses for two generations.

Because ε-PL is harmless to human and biodegradable, as well as its water-binding capability, ε-PL has been also used for the synthesis of hydro-gel by cross-linking of ε-PL and polysaccharides. The hydro-gel is already a commercial product applied in agriculture and medical industries[92].

5 Prospects in poly amino acids production

There are many biopolymers have been known, but only three kinds of them have been found in natural microorganism until now. Because of diversity of microorganisms in various environmental conditions, it is possible to identify more strains with high ability to produce poly amino acids and other biopolymers. Poly amino acids are useful biomaterial for their friendship to environment, high solubility and biodegrading ability. However, one big obstacle in their large-scale application is the rather high cost and low productivity. Further research should be done to make this kind of biopolymers to be produced effectively in a short cultivation time. The molecular weight and *D*/*L* ratio control of poly amino acid is also important in their production. Moreover, the chemical modified poly amino acid could be used as a recyclable material and deserve more attention.

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