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Preface

Biopolymers are the most abundant molecules in living matter. Microorganisms are capable of producing a wide variety of biopolymers, including polynucleotides, polyamides (protein), polysaccharides, polyphosphate, polyesters, and polyketides. However, homopolymers, which are made up of only a single type of amino acid, are far less ubiquitous; in fact, only two amino-acid homopolymers are known to occur in nature: poly-ε-L-lysine (ε-poly-L-lysine, ε-PL) and γ-poly-glutamic acid (γ-PGA).

ε-PL, consisting of 25–30 L-lysine residues with a linkage between the α-carboxyl group and the ε-amino group, is produced by actinomycetes. Because ε-PL is a polycationic peptide and thus exhibits antimicrobial activity against a wide spectrum of microorganisms, including Gram-positive and Gram-negative bacteria, and because it is both safe and biodegradable, ε-PL is used as a food preservative in several countries. In contrast, γ-PGA is an unusual anionic polypeptide in which D- and/or L-glutamate is polymerized via γ-amide linkages. γ-PGA is secreted into the growth medium of *Bacillus subtilis* as a fermentation product with a variable molecular weight (typically, 10–1,000 kDa).

Over the past decade, the biological and chemical functions of these two homopoly amino acids have been reported, thereby being promising materials for medical and industrial applications. This Microbiology Monographs volume covers the current knowledge and most recent advances in regard to the occurrence, biosynthetic mechanisms, biodegradations, and industrial and medical applications of these polymers.

Fukui, Japan — Yoshimitsu Hamano

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Occurrence and Production of Poly-Epsilon-L-Lysine in Microorganisms

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Abstract This chapter addresses the occurrence and production of poly-ε-L-lysine (ε-PL) in filamentous bacteria from the family Streptomycetaceae and ergot fungi, especially in the genus *Streptomyces*. The presence of ε-PL, first discovered from a strain among 2,000 actinomycetes, was found quite frequently in various strains of *Streptomyces* by novel screening methods, including the two-stage culture of cell growth and ε-PL production cultures. Using the newly isolated producer strains of *Streptomyces*, their production behaviors were studied not only in terms of the time course of several production factors and effect of culture medium components, but also other aspects of the release of synthesized ε-PL into the culture broth and of the simultaneous development of ε-PL hydrolase activity with the ε-PL-producing machinery. The ε-PLs obtained were evaluated structurally. The results revealed that the polymers had a nearly monodispersed structure, and could be classified into five groups based on their chain lengths. The cell density-dependent control of the production of ε-PL, the chain length shortening by aliphatic hydroxy-compounds, and the coproduction of novel amino acid homopolymers with ε-PL are also discussed.

1 Introduction

Poly-ε-L-lysine (ε-PL) (also called ε-poly-L-lysine) is an L-lysine linear homopolymer biosynthesized extracellularly, and has a unique structure linking ε-amino and α-carboxylic acid functional groups (Fig. 1). The polymer of 25–35 residues was discovered as a secreted product from a strain of *Streptomyces albulus* No. 346, now designated *S. albulus* NBRC 14147 (NBRC 14147), in culture filtrates (Shima and Sakai 1977). The compound is biodegradable and water soluble, and has various functions such as antimicrobial activity (Shima et al. 1984; Hiraki 2000), antiphage action (Shima et al. 1982), endotoxin-selective removal action (Hirayama et al. 1999), and antiobesity action due to the inhibition of pancreatic lipase (Tsujita et al. 2006). This polymer is practically nontoxic in acute, subchronic and chronic feeding studies in rats, and nonmutagenic in bacterial reversion assays (Hiraki et al. 2003). Since the discovery of NBRC 14147, the production of ε-PL has been enhanced nearly 100-fold through various optimization attempts in fermentation techniques such as strict controls of the pH and glucose concentration of culture media using a certain mutant of the first strain (Kahar et al. 2001). ε-PL is manufactured at the commercial scale by a fermentation process using the mutant of NBRC 14147, and is used as a food preservative in several countries (Oppermann-Sanio and Steinbüchel 2002; Yoshida and Nagasawa 2003).

Despite the fact that this polymer was scientifically so interesting and practically so useful, studies on ε-PL have been rather limited both in quantity, scope and the

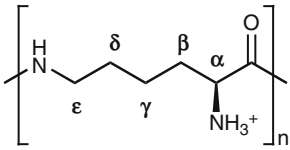

Fig. 1 Chemical structure of ε-poly-L-lysine (ε-PL) biosynthesized in microorganisms

level of detail examined as compared with poly-γ-glutamic acid (γ-PGA) (see chapter "Occurrence and Biosynthetic Mechanism of Poly-Gamma-Glutamic Acid" by Ashiuchi) or cyanophycin, the storage amino acid polymer which accumulates inside producing cells (Oppermann-Sanio and Steinbüchel 2002, 2003). This might be mainly attributed to the fact that ever since the first discovery of the *S. albulus* strain, no microorganisms producing ε-PL had been isolated until recently when two novel screening methods succeeded in isolating several strains of Streptomycetaceae and ergot fungi (Nishikawa and Ogawa 2002; Kito et al. 2002a). All of the specific properties mentioned above were studied using ε-PL samples from NBRC 14147 or its mutant. γ-PGA was discovered 40 years before ε-PL (Ivánovics and Erdös 1937), and many experiments have been performed on it over the years in various fields and levels. Cyanophycin, discovered in the nineteenth century, has also been well studied in terms of its biosynthesis at the molecular and biological levels (Oppermann-Sanio and Steinbüchel 2002, 2003).

Under these circumstances, the presence of ε-PL was found to be much more frequent than had been anticipated, through the screening of various actinomycete strains (Hirohara et al. 2007). Of the plus 200 strains found to produce cationic polymers, ten strains and their ε-PLs were studied in detail. All ten belonged to the genus *Streptomyces*. The authors examined the effects of the components of the culture medium on ε-PL production as well as the production behaviors in these strains (Hirohara et al. 2006). They reported the number of lysine residues (R_n), number and weight average molecular weight (M_n, M_w), and polydispersity index (M_w/M_n) of the polymers obtained from glycerol or glucose (Hirohara et al. 2007). They also studied how the ε-PL was released into the culture broth, and how the development of ε-PL-production and hydrolyzing activities were correlated in certain producer strains (Saimura et al. 2008). All of these reports will further facilitate the study of ε-PL in both fundamental research and technical applications by obtaining a variety of novel polymers with desirable polymeric structures.

This chapter gives an up-to-date overview on the occurrence and production of ε-PL in microorganisms. It includes the frequent occurrence of ε-PLs with various R_ns, the nearly monodispersed structures of ε-PLs irrespective of their R_ns, the control of production, shortening of the chain length through esterification, and the coproduction of another amino-acid homopolymer (poly(amino acid)) with ε-PL. The biosynthetic mechanism is not discussed here, since the genes and enzymes involved in the biosynthesis are discussed fully in chapter "Biochemistry and Enzymology of Poly-Epsilon-L-Lysine Biosynthesis" by Hamano.

2 Screening and Discovery of Poly-ε-L-Lysine Polymers

A quarter of a century after the discovery of the first producer strain, a dozen microorganisms have been found to produce the polymer using two novel and independent screening methods. Thereafter, the much more frequent presence of ε-PL than had been previously anticipated was supported by screenings of various

Table 1 Proton and ^{13}C NMR chemical shifts of ε-PL in D_2O at pD 2.7 (δ in ppm)

	αCH	α^{C}CH	βCH$_2$	γCH$_2$	δCH$_2$	δ^{N}CH$_2$	εCH$_2$	ε^{N}CH$_2$	C(=O)
$\delta^{1}H$	3.95	3.85	1.88	1.41	1.58	1.71	3.25	3.01	
$\delta^{13}C$	56.0		33.2	24.5	30.7		41.9		172.4

Recorded on a JEOL JMN-LA400 FT NMR spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The superscripted N or C denote the N- or C-terminal groups of the polymer. The ε-PL sample was obtained from strain USE-11

strains of *Streptomyces* employing two-stage culture methods. The structure of the polymer was identified by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) experiments (Table 1). This section will reveal that it is not difficult to obtain a strain producing a sufficient amount of ε-PL with a desirable chemical structure.

2.1 First Discovery as Dragendorff-Positive Substance

An attractive biopolymer, ε-PL, was discovered at first as a high molecular-weight compound secreted from a strain of *S. albulus* in the course of screening for Dragendorff-positive substances (i.e., alkaloids or quaternary nitrogen compounds) from approximately 2,000 actinomycetes (Shima and Sakai 1977, 1981a). The substance purified from the culture filtrates was identified as ε-PL by infrared spectra, paper chromatography, optical rotation, and chemical methods, and its degree of polymerization and the molecular weight were determined (Shima and Sakai 1981b). Since the discovery of the first producer and its sufficient production of ε-PL, it has been the sole material for the investigation of the polymer ever since.

2.2 Every Producer Strain Has ε-PL-Degrading Activity

Kito et al. (2002a) isolated an ε-PL-degrading enzyme from NBRC 14147, and suggested a correlation between high ε-PL-degrading activity and ε-PL-producing activity. It is known that a certain biopolymer is digested with a polymer-degrading enzyme(s) produced by its own host. Thus, it is not strange that every ε-PL producer strain also has high ε-PL-degrading activity. This was exemplified by the ε-PL-degrading activity in the membrane fraction of type culture strains of *S. virginiae* (NBRC 12827) and *S. norsei* (NBRC 15452). These two strains were demonstrated to produce 1.7 and 0.3 g l^{-1} of ε-PL in the culture media, respectively. We also observed all of the ε-PL producers examined also had ε-PL-hydrolyzing activity (Sect. 3).

As a result, screenings for ε-PL producer strains could be performed based on their ε-PL-degrading activity as a barometer of ε-PL-producing capability. This method deserves more attention, since it is applicable for type culture strains from

publicly accessible culture collections as screening targets, using a substrate with a specific chromophore such as L-lysyl-*p*-nitroanilide. This is convenient for chemists or biochemists who are rather hesitant to carry out screenings using soil samples. However, it should be noted that the ε-PL-degrading activity does not always show the presence of ε-PL-producing activity. Protease A originating from *Aspergillus oryzae*, for instance, has good ε-PL-degrading activity (Kito et al. 2002b), but this fungus does not produce ε-PL.

2.3 High Throughput Screening in Agar Plates

A simple screening method with an acidic polymeric dye, Poly R-478, succeeded in obtaining several ε-PL-producing microorganisms (Nishikawa and Ogawa 2002). This method detected the basic polymers that interacted with the charged dye embedded in the agar plate. Using a solid culture medium, it was possible to examine up to 100–300 colonies simultaneously on a single culture plate. From 300 soil samples, more than ten ε-PL-producing strains were found by this high throughput screening method. The chemical structures of the polymers were confirmed by thin-layer chromatography, and the R_ns of the polymer were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

The ε-PL-producers obtained were identified as from the genera of *Streptomyces*, *Streptoverticillum*, *Kitasatospora*, and *Epichloë*. The distribution of ε-PL producers was limited in the filamentous bacteria of the family Streptomycetaceae and ergot fungi. It is noteworthy that ε-PLs were produced by microorganisms separated by a large evolutionary distance, among which the biosynthetic genes might transfer horizontally. Despite the restricted distribution, there was structural diversity in the isolated ε-PLs with regard to the R_n. The molecular weights of the ε-PLs were reported to range between 800 and 2,000 from their MALDI-TOF mass spectra. A strain of *Claviceps purpurea*, an ergot fungus, was already known to produce basic proteins (clavicepamines) that contain ε-PL polymers as the fundamental structural units (Szókán et al. 1997).

2.4 Frequent Occurrences Found by Two-Stage Culture Method

The frequent presence of ε-PL was found in various strains of *Streptomyces* (Hirohara et al. 2007). The two-stage culture screening method for cell growth and ε-PL production cultures was applied to soil actinomycetes to obtain strains that secrete ε-PL. At the first stage, a loopful of each colony was inoculated into a test tube containing a growth culture medium, and was incubated for 20–48 h at pH 6.8 and 30°C (cell growth culture). At the second stage, the mycelia collected by centrifugation were resuspended with production medium, and cultured for up to

Table 2 Novel ε-PL producer strains of *Streptomyces* and their production levels (modified from Hirohara et al. 2007)

Producer strain	Abbreviation	Production level (g l^{-1})
Streptomyces lydicus USE-11	USE-11	4.0
Streptomyces sp. USE-12	USE-12	2.0
S. albulus subsp. USE-13	USE-13	2.5
S. albulus NBRC 14147[a]	NBRC 14147	2.8
S. celluloflavus USE-31	USE-31	0.8
S. celluloflavus subsp. USE-32	USE-32	0.5
Streptomyces sp. USE-33	USE-33	0.4
Streptomyces sp. USE-51	USE-51	0.8
S. herbaricolor USE-52	USE-52	0.5
S. lavendulae USE-81	USE-81	0.8
S. aureofaciens USE-82	USE-82	4.5

[a]The first strain discovered by Shima and Sakai (1977)

7 days at pH 4.5 and 30°C (ε-PL production culture). Glycerol was used as the carbon source in both screening cultures.

Of the 1,900 actinomycete colonies isolated on glycerol-Czapek plates, more than 200 colonies were found to give positive results on the Methyl Orange (MeO) precipitation test. All of the secretions from the 200 isolates seemed to be ε-PL, since their SDS–PAGE analysis gave broad bands within the range of molecular weight estimates of ε-PL (2,000–4,500). At the late period of the screening study, nearly 30% of the producer strains were obtained from soil samples under decayed, thick fallen leaves in the woods or forest. Among the 200 colonies, nearly 50 strains secreted fairly large amounts ($\geq$0.3 g l^{-1}) of ε-PL in their culture broths. Surprisingly, the occurrence of ε-PL was much more frequent than had been anticipated previously.

Among the 50 strains that produced large amounts of ε-PL, 10 strains along with the ε-PLs they produced were studied in detail. All ten strains were identified as the *Streptomyces* genus, and were designated as shown in Table 2, together with each production level. The ten strains were deposited in publicly accessible culture collections. The two-stage culture screening method was effective, and the MeO detection method was highly sensitive to ε-PL. It may not be difficult to obtain a desirable ε-PL producer from such soil samples as mentioned above.

3 Production Behavior in *Streptomyces* Strains

The production behavior of ε-PL is examined in this section using a few strains out of the ten newly isolated *Streptomyces* strains described in the preceding section. All examinations were performed using glycerol as the carbon source by the two-stage culture method. This method clearly differentiates between cell growth and ε-PL production stages, and is suitable for studying the production stage exclusively by separating it from the cell growth stage. In Sect. 3.4, however, a one-pot

fermentation method was employed to successively observe the cell growth and ε-PL production stages.

3.1 Features Shown by the Two Strains

The production features were studied in the two producer strains USE-11 and USE-51. The former synthesizes ε-PL with identical chain lengths with the NBRC 14147 polymer at high production levels, whereas the latter produces polymers with much shorter chain lengths than the former at a low level. The time course of the ε-PL produced, together with the glycerol, citrate, and pH levels of the medium, was illustrated using cells growth-cultured for 33 h in USE-11 and 25 h in USE-51 (Fig. 2a and b). The ε-PL production by all strains tested exhibited the common phenomenon of reduced polymer levels to zero after reaching the optimums, as shown in Fig. 2. Such a disappearance corresponded well with the pH increase from pH 4.5 to a neutral pH in the culture media. This pH increase accompanied by the disappearance of ε-PL was associated with the exhaustion of glycerol instead of citrate having its buffer action. These phenomena were also commonplace in all ε-PL-producing strains examined. It was shown with USE-51 that constant feeding with glycerol to compensate for the consumption maintained the production level, as well as the pH value (Fig. 3). This may indicate that the disappearance of the polymer was caused by digestion due to an ε-PL-hydrolyzing enzyme(s) produced by each ε-PL producer strain.

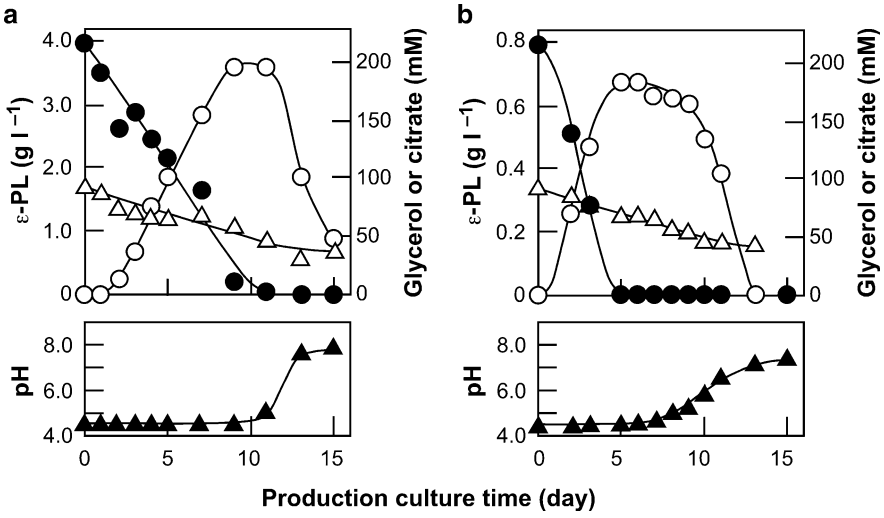

Fig. 2 Time course of ε-PL production (*open circle*), concentration of glycerol (*filled circle*) and citrate (*open triangle*), and pH of the culture medium (*filled triangle*) in: (**a**) USE-11 and (**b**) USE-51. The culture medium consisted initially of 76 mM $(NH_4)_2SO_4$ and 11 mM L-lysine·HCl in addition to glycerol and citrate (updated from Hirohara et al. 2006)

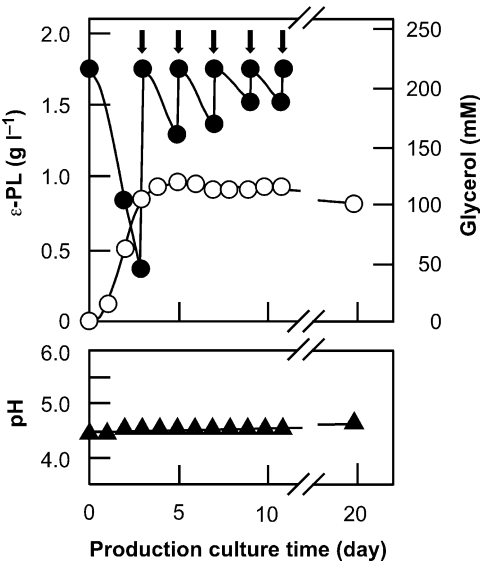

Fig. 3 ε-PL production (*open circle*), glycerol concentration (*filled circle*) and pH of the culture medium (*filled triangle*) in fed-batch culture of USE-51 cells growth-cultured for 25 h. *Arrows* indicate the addition of glycerol to maintain a concentration of 220 mM (Hirohara et al. 2006 (ESM-1))

It is not easy to obtain a plausible answer as to explain why the optimum level is maintained by the constant feeding of glycerol and continuous culture at pH 4.5. The hypothesis that the production and digestion of the polymer are balanced out in the culture medium may be easily ruled out by the fact that no ε-PL-hydrolyzing activity was detected at pH 4.5 as mentioned above.

3.2 *Effects of the Culture Medium*

As a nitrogen source, $(NH_4)_2SO_4$ yielded the best results of various nitrogen substances such as NH_4Cl, NH_4NO_3, $NaNO_3$, urea, casamino acid, polypeptone, or yeast extract for USE-11. This effect of $(NH_4)_2SO_4$ was also observed in many other *Streptomyces* producer strains, including NBRC 14147. The NH_4^+ form was the most effective nitrogen source, and the presence of SO_4^{2-} in the production culture medium was found to be critical for ε-PL synthesis in all strains examined.

Among the organic acids in the citric acid cycle (TCA cycle), citrate facilitated the production best and yielded the highest level of polymer, whereas succinate completely inhibited the polymer production in all strains examined. Other organic acids in the cycle such as α-ketoglutarate and malate were in-between in USE-11 or USE-51. This may be due to the fact that citrate facilitates the conversion of oxaloacetate to L-aspartate rather than the cycle-forming reaction to citrate. Thus, it is desirable to add citrate to the production medium. In USE-82, however, the addition of citrate, malate, or α-ketoglutarate to the production culture medium equally enhanced the production of ε-PL. This effect of α-ketoglutarate suggests

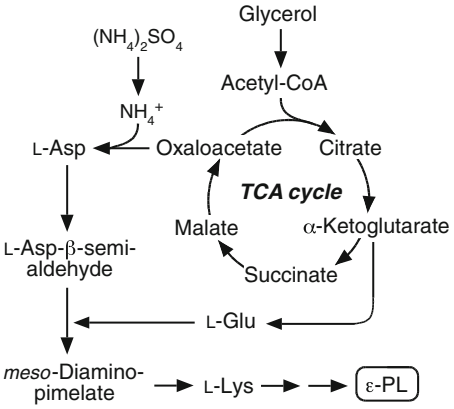

Fig. 4 Schematic representation of the putative pathway for ε-PL synthesis in *Streptomyces* strains

that the flux in the TCA cycle may diverge to L-glutamate to such an extent that it may combine with L-aspartate-β-semialdehyde to generate *meso*-diaminopimelate (Fig. 4). The effects of malate might indicate that this organic acid facilitates the syntheses of both L-aspartate and L-glutamate.

In media consisting of citrate, glycerol, and $(NH_4)_2SO_4$, the addition of 11 mM L-lysine gave positive effects on the optimum production level of ε-PL in USE-11. However, no effects were observed on ε-PL production in USE-51. An ample supply of 110 mM L-lysine caused a slight decrease in the optimum ε-PL production in USE-11, whereas a great decrease to less than one-tenth of the original level was observed in USE-51. It is known in various bacteria, including the *Streptomyces* genus, that L-lysine, an end product of primary metabolism, effectively regulates aspartokinase, the first enzyme in the diaminopimelic acid pathway from L-aspartate to L-lysine, through feedback inhibition. The great production of ε-PL indicates that the enzyme in USE-11 might be resistant to feedback inhibition from L-lysine to a considerable extent, as was recently demonstrated in NBRC 14147 (Hamano et al. 2007). D-Lysine showed strong inhibitory effects in all the strains tested, and no D-isomer was incorporated into ε-PL.

3.3 Release of Polymers into the Culture Broth

One of the most interesting questions to be answered for an extracellular biopolymer is how the synthesized molecules are released into culture broth. As an attempt to answer this question with ε-PL, Saimura et al. (2008) measured the amount of ε-PL that had accumulated in cells from the beginning of a production culture using USE-11, and compared these amounts with those from polymers secreted into the culture broth. The results are shown in Fig. 5 as time courses of ε-PL accumulation.

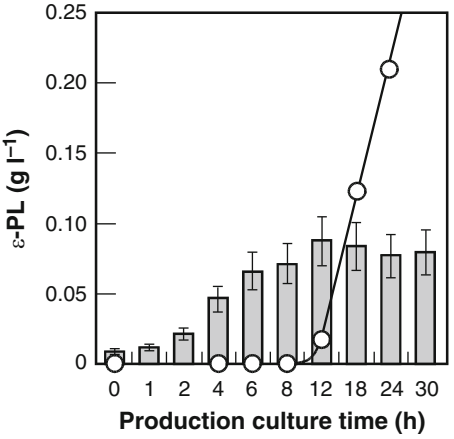

Fig. 5 Time course of ε-PL accumulation in the cells (*filled box*) and culture broth (*open circle*) of USE-11 (Saimura et al. 2008)

The production of ε-PL began with the production culture, but the release into the culture broth had a threshold level (Fig. 5). The level was very low (only 2–2.5%) as compared with the observed optimum production level of 4.0 g l^{-1}. This implies that almost all of the ε-PL molecules produced in the cell were released into the broth immediately after production. In this context, an interesting result was observed that almost 100% of the ε-PL in the cells could be washed out with 3 M NaCl. This suggests that the elongating polymer chains passed through the pore of an integral membrane protein outside of the cells, and that the polymer segments were already present outside of the cells when the elongating intermediates were terminated by a nucleophilic chain transfer agent. Since cellular surfaces are negatively charged, the terminated ε-PL molecules may first remain on the cellular surfaces via electrostatic interactions, and then the continuously produced ε-PL molecules overflow into the culture broth.

3.4 Development of ε-PL-Hydrolyzing Activity

Every ε-PL producer strain had ε-PL-degrading enzyme activity (sect. 2.2). The ε-PL secreted was digested in a neutral pH range by an ε-PL-hydrolyzing enzyme produced by its own producer strains (sect. 3.1). Thus, we examined the correlations between the development of ε-PL-hydrolyzing activity and the production of ε-PL with USE-11 (Fig. 6) (Saimura et al. 2008). The one pot fermentation method was employed to facilitate the earlier development of both ε-PL-producing and ε-PL hydrolase activities than the two-stage culture method, as well as to observe successive cell growth and ε-PL production stages. Both activities began to develop at 24-h postfermentation, immediately after the medium pH spontaneously declined

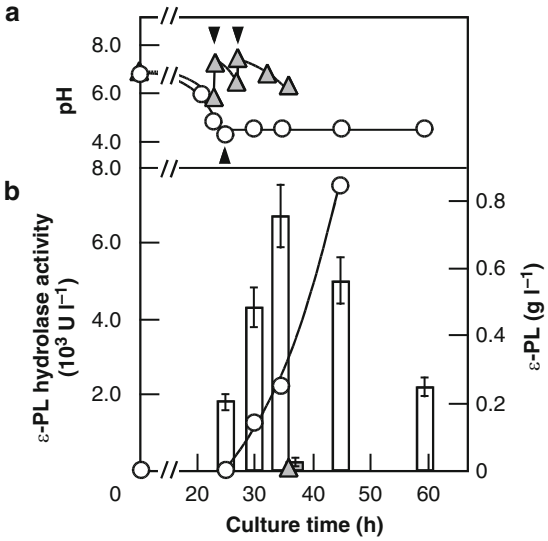

Fig. 6 Time courses of pH of the culture media, ε-PL productions at pH 4.5 (*open circle*) and pH 6–7 (*filled triangle*), and ε-PL hydrolase activity at pH 4.5 (*open box*) and pH 6–7 (*filled box*) in USE-11 during a one pot fermentation process. (**a**) The pH value decreased spontaneously to produce the polymer. By feeding citrate buffer (*upward arrowhead*), the value was maintained at pH 4.5 (*open circle*) so as not to decrease further, whereas by adding NaOH (*downward arrowhead*), the pH value was kept at 6–7 (*filled triangle*). (**b**) The ε-PL production and ε-PL hydrolase activities were determined from each culture medium (updated from Saimura et al. 2008)

to 4.5 to produce the polymer. The ε-PL hydrolase activity increased simultaneously until 35–40-h postfermentation while the pH value was maintained at 4.5. On the other hand, while the pH was intentionally kept at 6–7, the USE-11 cells did not produce any ε-PL, and the hydrolase activity detected was negligible. Thus, it appears that the presence of the ε-PL molecules causes the development of the hydrolase activity. However, the addition of the ε-PL polymer to the medium did not activate the hydrolase activity over a neutral pH range. It is therefore plausible that the operation of the ε-PL-producing machinery induces the hydrolase activity when the medium pH is maintained around 4.5. The activated hydrolase might be an ε-PL specific hydrolyzing enzyme directly associated with ε-PL production in USE-11.

A decrease in the hydrolase activity was detected at 46-h postfermentation and further declines were observed at 59 h (Fig. 6b). This decline might be due to the fact that the ε-PL specific hydrolase was digested by a protease(s), capable of acting at pH 4.5, secreted into the culture broth independently of ε-PL. It is known that the *Streptomyces* genus produces a variety of extracellular proteases. The ε-PL hydrolase in USE-11 may be an anchored or a peripheral membrane protein on the outside of the cells, since ε-PL was digested only when the polymer solution was kept in contact with the cultured cells.

4 Polymer Structure of ε-PL in *Streptomyces* Strains

We evaluated the polymer structures, i.e., the R_n, M_n, and M_w/M_n of the ε-PLs produced by the ten newly isolated *Streptomyces* strains, along with those from NBRC 14147, using ion-pair high performance liquid chromatography (ion-pair HPLC) (Hirohara et al. 2007). The R_n of the NBRC 14147 ε-PL has been measured by MALDI-TOF MS (Nishikawa and Ogawa 2006). Glycerol or other aliphatic hydroxy-compounds were found to reduce the R_n by C-terminal esterification. MALDI-TOF MS is a powerful technique for the structural characterization of biomolecules and polymers. However, the spectral intensities for molecules with high molecular weights greater than 10^3 were demonstrated to decrease with an increase in molecular weight (Shimada et al. 2003). This problem is too important to be neglected for molecules with a molecular weight distribution such as ε-PL, and attempts to overcome this problem have been still continued (Nagahata et al. 2007; Schlosser et al. 2009). Thus, we employed the ion-pair HPLC method for estimating the M_n and M_w of the ε-PLs. The method is based on the number of charged amino groups, and thus the determination of the M_n and M_w by this method is reliable so long as baseline separation is maintained.

The R_n, M_n, and M_w/M_n values of the 11 ε-PLs produced by the new strains and NBRC 14147 using the two-stage culture method are summarized in Table 3. The ε-PLs could be classified into five groups according to their R_ns. The groups were designated as shown in the second column of the Table in order of R_n for the convenience of discussion below. Figure 7 shows ion-pair chromatograms of ε-PLs from both 2% glycerol and 2% glucose in the five groups. It should be noted that the largest R_n from glucose was unchanged by the use of glycerol in all of the strains examined, except for USE-33 (Fig. 7, Table 3). The average R_n from glucose in each group was 32, 28, 25, 19, and 16 from the top, respectively. Thirty-six was the longest chain length found so far. These numbers, apparently multiples of 4, might

Table 3 Characterization of ε-PLs produced from glycerol or glucose[a] (updated from Hirohara et al. 2007)

Producer strain	Group	R_n[b]		M_n[b]		M_w/M_n[b]	
		Glycerol	Glucose	Glycerol	Glucose	Glycerol	Glucose
USE-11	A	15–35	24–36	3,500 ± 60	4,050 ± 10	1.03	1.01
USE-12		14–35	22–35	3,500 ± 80	3,920 ± 10	1.03	1.01
USE-13		10–36	–	3,500 ± 20	–	1.03	–
NBRC 14147		14–35	22–36	3,450 ± 60	3,960 ± 10	1.05	1.01
USE-33	B	8–32	12–35	2,920 ± 50	3,600 ± 30	1.06	1.04
USE-31	C	12–29	17–29	2,840 ± 60	3,140 ± 10	1.03	1.01
USE-32		10–29	17–28	2,720 ± 70	3,110 ± 10	1.03	1.01
USE-51	D	10–23	13–23	2,150 ± 10	2,390 ± 10	1.03	1.01
USE-52		10–23	13–23	2,150 ± 30	2,390 ± 10	1.03	1.01
USE-81	E	8–19	10–20	1,670 ± 20	2,000 ± 10	1.03	1.02
USE-82		5–20	10–21	1,680 ± 60	2,080 ± 20	1.06	1.02

[a]The initial concentration was 2% (w/v) for both carbon sources
[b]Determined from ion-pair chromatograms

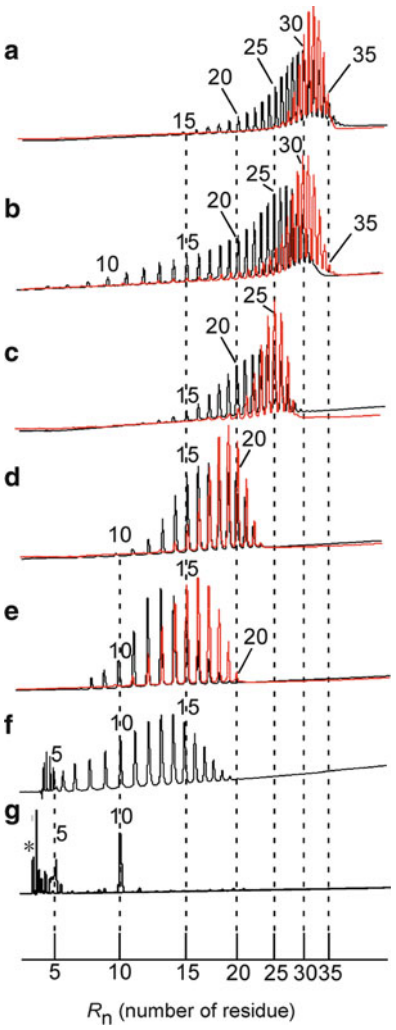

Fig. 7 Ion-pair chromatograms from the HPLC analysis of ε-PL hydrochlorides (ε-PL·HCls) produced from glucose (*red*) or glycerol (*black*). The polymers were produced by the strains: (**a**) USE-11, (**b**) USE-33, (**c**) USE-31, (**d**) USE-51, (**e**) USE-81, (**f**) partially hydrolyzed ε-PL·HCl secreted by USE-82 and (**g**) chemically synthesized ε-L-lysine oligomers·HCl consisting of 5 or 10 residues (*asterisk* indicates impurity peaks)

reflect different varieties in the ε-PL synthetic mechanism or the subunit structure of the ε-PL-synthesizing enzyme in the cell membrane.

The carbon source had a remarkable effect on the molecular weights and M_w/M_n ratios of the ε-PLs. Glucose yielded nearly monodispersed ε-PLs in most of the strains. ε-PL is the first poly(amino acid) that showed monodispersity, which is one of the most desirable characteristics in a polymeric compound, and is critical for determining the relationship between the molecular weight and its function. All of the ε-PLs from 2% glycerol had 10–20% lower M_n values and a slightly broader M_w/M_n ratio than those from 2% glucose, but still showed a fairly narrow molecular weight distribution. The molecular weights of the polymers were neither changed by the culture time nor the culture medium composition other than the carbon

source. These results indicate that the molecular weight and polydispersity index of ε-PL were primarily determined by each producer strain.

5 Two Advantageous Producers

The first strain NBRC 14147 and its product have been used exclusively thus far for fundamental research and the application studies of ε-PL. This may be partly because of the high yield of ε-PL in this strain, and partly because of the advantageous polymer structure of the ε-PL produced. This section describes the merits of two high yield producers out of the ten *Streptomyces* strains discussed in the previous sections. These two strains also produce ε-PLs no less advantageous and useful than the NBRC 14147 ε-PL for the fundamental studies or the application aspects of the polymers.

5.1 Streptomyces lydicus *USE-11*

USE-11 yields ε-PL classified into group A (Table 3) at a high yet stable production level, irrespective of the cell growth culture time. Experimental results suggested that this strain had great metabolic fluxes in ε-PL synthesis as well as L-lysine supply in the cells (Hirohara et al. 2006). The strain not only showed high productivity, but also produced an ε-PL-hydrolyzing enzyme with great activity, and further enabled us to simply purify and isolate the ε-PL polymer, because ε-PL was the major compound among the excreted peptidyl compounds in the culture medium. Taking these advantages into consideration, the reason why and how the synthesized ε-PL molecules were released into the culture broth and the development of ε-PL specific hydrolase activity were investigated (Saimura et al. 2008). The results are described in Sect. 3. In addition, the lack of any control over the production system in this strain, as is discussed in the following section, might help with the isolation of both ε-PL-synthesizing and ε-PL-degrading enzymes, and facilitate the cloning of the encoding genes. We emphasize that USE-11 is the most useful and advantageous producer strain from the viewpoints of fundamental research as well as technical applications.

5.2 Streptomyces aureofaciens *USE-82*

USE-82 also produced the highest level of ε-PL of all producer strains examined (Takehara et al. 2010). The average R_n of the polymer classified into group E was one-half of that from NBRC 14147. Since ε-PL with more than ten lysine residues has optimal antimicrobial activity (Shima et al. 1984), the use of the USE-82 ε-PL

as a food preservative may be attractive. It also might reduce the bitter taste characteristic of L-lysine residues, and improve the taste of ε-PL for consumption.

It is interesting to understand how USE-82 produces ε-PL at high production levels, despite its short chain length, and how the strain produces short chain lengths of ε-PL. USE-82 might have great metabolic fluxes in both the L-lysine supply from the citric acid cycle and ε-PL synthesis from L-lysine just as USE-11 had. Quite recently, an ε-PL-synthesizing enzyme was identified for the first time from NBRC 14147 (Yamanaka et al. 2008). However, it is still unclear how the chain termination occurs during the synthesis of ε-PL. Thus, USE-82 might be a valuable research target to elucidate the mechanism underlying the termination mode.

6 Production Control and Chain Length Shortening

This section deals with the cell density-dependent production of ε-PL with short chain lengths, in group D (Table 3), and the chain length shortening of the polymer by hydroxy-compounds via esterification at the C-terminus of ε-PL. It may be useful to discuss such phenomena for the further investigation of ε-PL in both fundamental research and technical applications.

6.1 Cell Density-Dependent Production

The production of ε-PL in USE-51 was found to depend strongly upon the time used for cell growth culture in the two-stage culture method for clearly differentiating between cell growth and ε-PL production (Hirohara et al. 2006). The 25-h growth-cultured cells gave the highest production level among all of the cells growth-cultured from 22 to 36 h. An extremely low level of ε-PL was produced with the 22-h growth-cultured cells, and no polymer was detected with the 36-h cultured cells. Cell growth culture time normally reflects the phase of cell proliferation, and hence the cell density. After monitoring the time courses for the CFU number and cell-density during cell growth culture, we examined the optimum production level of ε-PL against the cell density with USE-51 and USE-11 (Fig. 8). The former strain yields ε-PL with short chain lengths on group D at a low production level, whereas in the latter strain, the polymer had twofold longer chains than the former ε-PL at a high production level (Tables 2 and 3). The production level in USE-51 was strongly dependent upon the cell density, whereas USE-11 produced ε-PL belonging to group A at high production levels, independent of the cell density. A very similar result to the former was also observed in USE-52, another low yield producer of short chain length ε-PL belonging to group D.

Cell density-dependent phenomena have been reported in the production of secondary metabolites such as antibiotics or morphological differentiation in

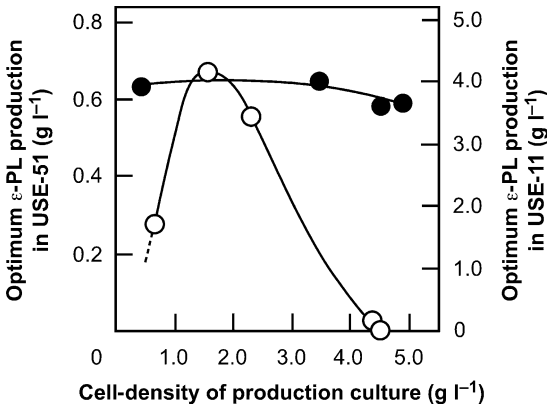

Fig. 8 Effects of cell-density of production culture on the optimum production level of ε-PL in USE-51 (*open circle*) and USE-11 (*filled cirlce*)

Streptomyces (Kleerebezem and Quadri 2001; Núñez et al. 2003). These phenomena are known as quorum sensing, which controls gene expression in response to cell-density by cells communicating with each other by means of certain chemicals (March and Bentley 2004). A similar system might operate for short chain ε-PL production in the above mentioned producer strains. Further studies are needed on this interesting phenomenon concerning the production of ε-PL.

6.2 Chain Length Shortening by Aliphatic Hydroxy-compounds

Nishikawa and Ogawa (2006) reported that the chain length of ε-PL was shortened by the use of short-chain aliphatic hydroxy-compounds, including glycerol, as carbon sources. They showed by MALDI-TOF MS and ^{13}C NMR spectroscopy analyses that a hydroxy group in the compound formed ester linkage with the terminal carboxyl group of the elongating ε-PL using ε-PL polymers produced in NBRC 14147 or a Streptomycetaceae bacterium, *Kitasatospora kifunense* MN-1. Glycerol had a weak potential to terminate the elongation of ε-PL, whereas 1,5-pentanediol showed the strongest effect on the shortening of the R_n among the compounds examined. The R_n of the ester decreased with increasing hydroxy-compound concentration. MALDI-TOF MS analysis revealed that the R_n of NBRC 14147 ε-PL decreased to 13–28 residues in the presence of 2.5% 1,5-pentanediol from 24 to 35 residues in the absence of the compound in the culture media. Incidentally, the R_n of the polymer thus shortened was rather similar to the values of ε-PL in group D (Table 3). Five percent 1,5-pentanediol completely inhibited polymer production without inhibiting cell growth. The authors also reported that almost 100% of the ε-PL released into the culture media of both NBRC 14147 and *K. kifunense* MN-1 appeared to be esterified with glycerol when the polymer was produced from 5% glycerol.

It is known that ^{13}C NMR spectra are not susceptible to quantitative analysis. Remarkable and nonquantitative effects have been reported in the MALDI-TOF

mass spectra of a polymer with a molecular distribution greater than 10^3 (Shimada et al. 2003). In addition to these findings, the observation that the greatest R_n of the polymer from glycerol was identical to that from glucose in almost all of the strains examined (Sect. 4) encouraged us to conduct a quantitative analysis of C-terminal ε-PL-glycerol ester using its ^{1}H NMR spectrum. We assigned resonances corresponding to the esterified C terminus α-proton (δ in ppm = 4.21) and carboxyl terminus α-proton (δ = 3.85) in the expanded 600 MHz ^{1}H NMR spectra of the ε-PL polymer. The percentages of the ester were evaluated to be 9, 15, and 15% in USE-11, NBRC 14147, and USE-33, respectively, from the relative integrated area of the peaks. Furthermore, it should be noted that no signals of the esterified C terminus α-proton were observed for polymers with short chain lengths produced in USE-51 or USE-82 (Hirohara et al. 2007), despite the fact that a shortening of the R_n of ε-PLs from glycerol was observed in these two strains (Table 3). Nishikawa (2009) reported that oligomeric ε-PL molecules produced in the presence of 1-octanol were not esterified by this alcohol, which had a strong ability to shorten ε-PL molecules to between 4-mers and 7-mers. These results clearly indicate that the chain length shortening is not always caused by esterification.

6.3 Chain Length Shortening Assisted by Sulfated β-Cyclodextrin

The use of aliphatic hydroxy-compounds such as 1,5-pentanediol may be unsuitable as a food-related product, despite their strong effects on the shortening of the ε-PL chain length. However, glycerol, generally regarded as safe for food, had little effect on the shortening of the chain length. An attempt was made to reduce the R_n of ε-PL by adding a β-cyclodextrin (β-CD) derivative to culture medium containing glycerol as a carbon source (Nishikawa 2009). When less than 1% β-CD sulfated in the high portions out of 21 hydroxy groups was added to the culture medium of *Streptomyces mashuense* MN-6 (Nishikawa and Ogawa 2002), all of the ε-PL molecules were esterified by glycerol at the C-terminus. The average R_n of the obtained polymer, evaluated by their MALDI-TOF mass spectra, was decreased from 30.5 with 5% glucose to 12.9 with 0.6% sulfated β-CD and 5% glycerol. Neither the sulfated β-CD alone nor unmodified β-CD along with glycerol showed such an effect on chain shortening.

The above observations might be suitable for a food-related use of ε-PL. The author reported that the polyanionic β-CD derivative might reinforce the action of amphiphilic glycerol, and interact with the nascent ε-PL chains generated by an ε-PL synthesizing enzyme to form a polyion complex between the sulfated β-CD and ε-PL. It may be pointed out that USE-81 or USE-82 gave nearly monodispersed ε-PLs with similar chain lengths (Table 3) to the polymer with the assistance of the β-CD derivative, and that it is easy to obtain partially hydrolyzed ε-PLs with short chain lengths as shown in Fig. 7f. However, the author argued that the above shortening method was superior in energy efficiency to enzymatic or chemical methods degrading ε-PLs.

7 Poly(Amino Acid) Coproduced with ε-PL

It is commonplace that *Streptomyces* species produce multiple secondary metabolites such as antibiotics and other biologically active compounds. However, it is not known whether such metabolites represent two distinct polymeric compounds. Two research groups found particular *Streptomyces* strains known as ε-PL producers which secreted two kinds of novel poly(amino acid)s along with ε-PL into the culture broths. This section reviews each of these two novel biopolymers coproduced with ε-PL.

7.1 Poly-γ-L-Diaminobutanoic Acid

The two strains of USE-31 and USE-32 out of the ten ε-PL producers described in the previous sections were found to secrete a novel polymeric compound, together with ε-PL, into their culture broths. The compound was identified as poly-γ-L-diaminobutanoic acid (γ-PAB), an L-α,γ-diaminobutanoic acid (L-DAB) linear homopolymer linking γ-amino and α-carboxylic acid functional groups (Fig. 9a) by amino-acid and chiral HPLC analyses, as well as one- and two-dimensional ^{1}H and ^{13}C NMR experiments (Takehara et al. 2008). Both strains coproduced high yields of the two poly(amino acid)s in the presence of SO_4^{2-} at pH 4.0 under sufficient aeration in a mini-jar fermentor. γ-PAB may be regarded as the third amino acid homopolymer occurring in nature.[1] The average R_n and M_n of γ-PABs produced by these two strains were estimated from the NMR signal area ratios of

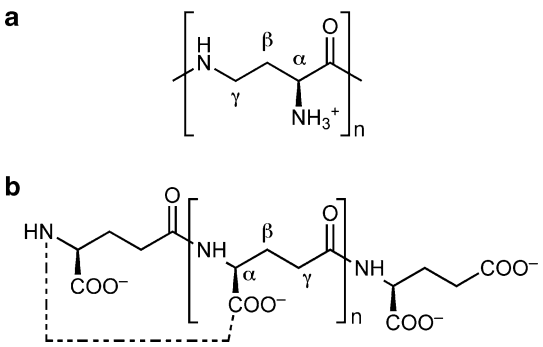

Fig. 9 Chemical structure of: (**a**) poly-γ-L-diaminobutanoic acid and (**b**) lariat-shaped poly-γ-L-glutamic acid coproduced with ε-PL in *Streptomyces* strains. The latter is given as the most probable structure

[1]We obtained a publication (Ohkuma et al. 1988) on an interesting biopolymer found as a novel antiviral agent, produced by an actinomycete (ATCC 31158), which was identified as poly-γ-D-diaminobutanoic acid. This polymer had a $M_n = 5{,}700$ and might actually be the third poly(amino acid) discovered in nature.

Table 4 R_n and M_n values of γ-PAB coproduced with ε-PL by two *Streptomyces* strains (modified from Takehara et al. 2008)

	Strain	
	USE-31	USE-32
Average R_n[a]	21.2 ± 1.9	20.7 ± 0.8
Average R_n of ε-PL[b]	22.0 ± 0.5	21.1 ± 0.5
M_n[a]	2,140 ± 190	2,090 ± 80
M_n[c]	2,200 ± 40	2,200 ± 40

[a]From the NMR signal area ratios of the internal to terminal α-protons in the range of pD 1.8–7.2
[b]Coproduced ε-PL (Hirohara et al. 2007)
[c]Estimated by gel filtration HPLC

the internal to terminal α-protons, as well as by gel filtration HPLC. The results are shown in Table 4, along with the average R_n values of the ε-PLs coproduced. The γ-PABs from these two strains had an almost identical polymer structure with each other, and the R_n values of the γ-PAB and ε-PL coproduced happened to be similar to each other within experimental error, although the significance of the similarity is not clear at present.

L-DAB is known as a precursor of the siderophores and a primary metabolite in some Gram-negative bacteria (Wang et al. 1996; Ikai and Yamamoto 1997) and is formed from L-aspartate β-semialdehyde and L-glutamate (Vandenende et al. 2004). It may be reasonable to hypothesize that L-DAB monomer molecules in both USE-31 and USE-32 are synthesized in a similar manner to this. No copolymers composed of the two amino acids L-DAB and L-lysine were found in either of the broths from the two producers. This might indicate that these two amino acids are polymerized by different enzymes, even if they were both generated by similar machinery.

From its similarity to ε-PL in chemical structure, γ-PAB can be regarded as a potential candidate for specific, advanced materials for technical applications in various fields just like ε-PL. Both γ-PAB and ε-PL have antibiotic antimicrobial activity, but show somewhat different spectra from each other in terms of activity. γ-PAB exhibited stronger inhibitory activities against various yeasts but slightly weaker actions against bacteria than ε-PL. The use of γ-PAB along with ε-PL might be more advantageous in exploiting their specific functions than their separate usage. It would be interesting to study whether they have synergistic actions, or only broader spectra, against biological targets by using them together, since antibiotics in general act synergistically against biological competitors (Challis and Hopwood 2003).

7.2 Lariat-Shaped Poly-γ-L-Glutamic Acid

A strain of *Streptomyces roseoverticillatus* previously isolated as an ε-PL producer via high throughput screening with Poly R-478 (Nishikawa and Ogawa 2002) was

observed to secrete an acidic substance into its culture filtrate (Nishikawa and Kobayashi 2009). GC/MS and HPLC analyses revealed that the substance coproduced with ε-PL was a mixture of L-glutamic acid oligomers consisting of 10–13 residues linking γ-carboxylic acid and α-amino functional groups. MALDI-TOF mass spectra indicated that the poly-γ-L-glutamic acid dehydrated to form a circular structure in the molecule (Fig. 9b), different from the known γ-PGA produced by *Bacillus* species. This novel polymer, designated lariat-shaped γ-PGA, is useful for controlling ε-PL dispersion by forming a polyion complex between the polymers. However, no gene for the biosynthesis of the lariat-shaped-γ-PGA was found in the region of that for ε-PL biosynthesis, despite the apparent correlation between the two polymers. Furthermore, the glutamic acid oligomer was produced by a disrupted mutant of the ε-PL biosynthesis gene. This strain might therefore be rather useful to study the initial stage of γ-PGA biosynthesis.

8 Concluding Remarks

Recent studies on ε-PL have resulted in sufficient knowledge on the occurrence and production of this polymer, especially its presence. Novel screening methods have revealed that ε-PLs are frequently present in *Streptomyces* strains to a great extent. Therefore, we may obtain, without great difficulty, ε-PL polymers with a variety of chain lengths. Using these ε-PLs and strains, valuable knowledge has also accumulated on the polymer structure of ε-PL, as well as various aspects of its production behavior. This knowledge will certainly contribute to the future exploration of ε-PL in not only fundamental research, but also in technical applications.

However, further studies are obviously needed to address unresolved problems. One of the most desirable occurrences may be the discovery and isolation of ε-PLs with R_ns values of more than 36. A polymer with a great degree of polymerization of over about 50 generally shows definite functions as polymer. It has been reported that chemically synthesized ε-PL with an R_n 40–200 showed antitumor activity in vitro and in vivo (Szókán et al. 1997). Another interesting phenomenon to be addressed would be the cell density-dependent production observed in the two short chain length ε-PL producers with low yields. It may be worthwhile for the purposes of further analysis on production to clarify such a phenomenon. Another issue may be chain length shortening. Further studies on this may elucidate the chain termination mechanism of the growing ε-PL polymer, which might give a hint as to how to produce ε-PLs with R_n values greater than 36. The other would be studies on the coproduction of novel poly(amino acid)s with ε-PL. All of these would greatly contribute to an analysis of the ε-PL production process, including the elucidation of the production machinery.

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Biochemistry and Enzymology of Poly-Epsilon-L-Lysine Biosynthesis

Yoshimitsu Hamano

Contents

Abstract *Streptomyces albulus* NBRC14147 (previously named IFO14147) is known to produce the amino-acid homopolymer antibiotic, poly-ε-L-lysine (ε-poly-L-lysine, ε-PL), consisting of 25–35 L-lysine residues with a linkage between the α-carboxyl group and the ε-amino group. Because ε-PL exhibits antimicrobial activity against a wide spectrum of microorganisms, including Gram-positive and Gram-negative bacteria, as well as antiphage activity, and because it is both safe and biodegradable, ε-PL has been introduced as a food preservative in Japan, South Korea,

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the United States, and other countries. This chapter covers the current knowledge and most recent advances in regard to the genetic system for *S. albulus* NBRC14147 and ε-PL synthetase.

1 Introduction

Streptomyces strains are known for their ability to synthesize commercially useful secondary metabolites having a wide range of biological activities. *Streptomyces albulus* NBRC14147 (previously named IFO14147) is known to produce the amino-acid homopolymer antibiotic, ε-PL, consisting of 25–35 L-lysine residues with a linkage between the α-carboxyl group and the ε-amino group (Fig. 1) (Shima and Sakai 1977; Shima and Sakai 1981a; Shima and Sakai 1981b). Because ε-PL exhibits antimicrobial activity against a wide spectrum of microorganisms, including Gram-positive and Gram-negative bacteria (Shima et al. 1984), as well as antiphage activity (Shima et al. 1982), and because it is both safe and biodegradable, ε-PL has been introduced as a food preservative in Japan, South Korea, the United States, and other countries.

The biological activity of ε-PL is known to be dependent on its molecular size. Shima and coworkers investigated the relationship between the molecular size of ε-PL and its antimicrobial activity against *Escherichia coli* K-12 (Shima et al. 1984). ε-PL with more than nine L-lysine residues severely inhibited microbial growth; however, the L-lysine octamer demonstrated negligible antimicrobial activity. In contrast, chemically synthesized α-poly-L-lysine that contains a considerably longer chain of L-lysine residues (50 residues), which show linkages between the α-carboxyl and α-amino groups, demonstrates a lower activity than ε-PL. Thus,

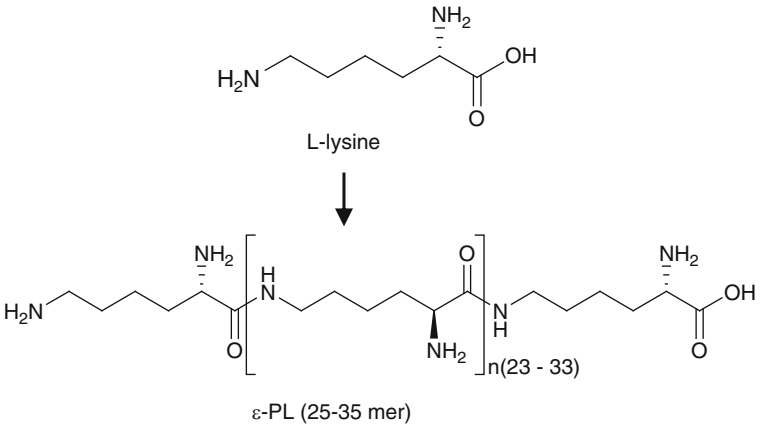

Fig. 1 Chemical structures of L-lysine and ε-PL (25-mer to 35-mer)

polymerization of L-lysine via an isopeptide bond is required to exert its biological activity, and the polymerization mechanisms involved in the chain-length diversity of ε-PL are of particular interest.

Investigation of an enzyme synthesizing ε-PL should facilitate biosynthetic engineering and help to create new classes of biopolymers. This review focuses on characterization of an ε-PL synthetase (Pls) and its biological machinery for ε-PL synthesis. In addition, an overview of effective genetic system for an ε-PL producer, *S. albulus* NBRC14147, which was used as a powerful tool for developing a deeper understanding of the Pls and for constructing an ε-PL overproducer, will be given.

2 Genetic System in an ε-PL Producer, *S. albulus* NBRC14147

Among the various gene transfer methods, polyethylene glycol (PEG)-mediated protoplast transformation is the standard for *Streptomyces* strains. In the initial PGE-mediated method transformation experiments on *S. albulus* NBRC14147, which were described for *Streptomyces lividans* (Kieser et al. 2000) and which used the typical cloning vectors having the pIJ101, pSG5, or pRES replicon (Kieser et al. 2000), no transformants were obtained. This unsuccessful outcome could be attributed to either or both of these reasons: (1) the vectors employed do not work in this strain; (2) the transformation methods are not suitable for direct application to the present strain of *S. albulus*. This section provides a brief overview of effective genetic system developed by Hamano et al.

2.1 Identification of the Cryptic-Plasmid pNO33 Replicon

A novel plasmid, pNO33, was detected in *S. albulus* NBRC14147 (Takagi et al. 2000). This large plasmid (37 kb) is a cryptic plasmid (Fig. 2), as none of its functions are yet known. To construct a cloning vector that can work in the *S. albulus* strain, the replicon (*Bcl*I–*Bam*HI 4.1 kb fragment, Fig. 2) of the cryptic plasmid pNO33 was used (Hamano et al. 2005). A circular plasmid carrying the *Bcl*I–*Bam*HI 4.1 kb fragment with the antibiotic (thiostrepton) resistant gene worked as a plasmid vector in *S. lividans* TK23, indicating that this plasmid (pBBH4) would also functionally operate as a replicon in *S. albulus*.

A database search with BLAST showed that the nucleotide sequence of this replicon had no similarity with those of the known replicons for cloning vectors, including pIJ702, which has the pIJ101 replicon and is frequently used in *Streptomyces* strains. Hamano et al. also reported that pBBH4 and pIJ702 are compatible for replication in the same cell of *S. lividans* (Hamano et al. 2005).

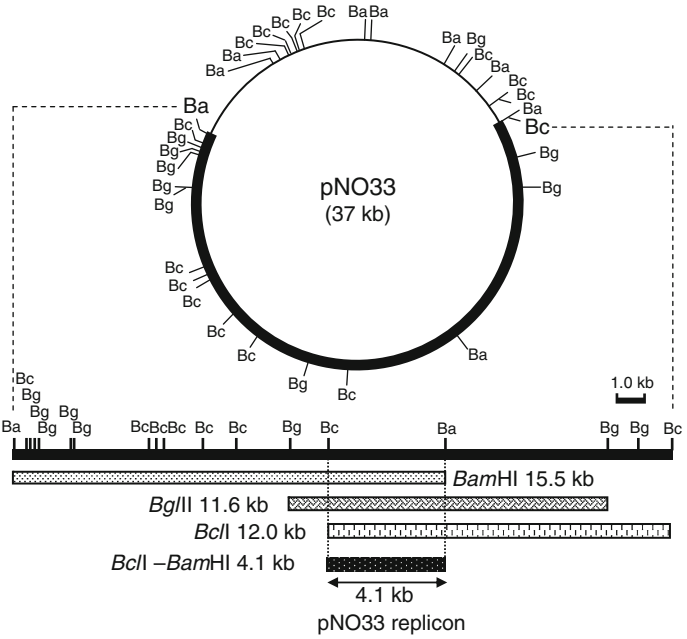

Fig. 2 Partial restriction map of pNO33 and schematic organization of the cloned fragment. *Bam*HI (15.5 kb), *Bgl*II (11.6 kb), and *Bcl*I (12 kb) fragments were cloned with a thiostrepton resistant gene (*Bcl*I 1.1 kb) in *S. lividans* TK23, demonstrating that these DNA fragments work as plasmid. Therefore, the pNO33 replicon is found to be located on the overlapping DNA fragment (*Bcl*I–*Bam*HI 4.1 kb). Abbreviations: Ba, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II

2.2 *Construction of the pNO33-Based Shuttle Vectors for* E. coli *and* Streptomyces *Strains*

Two cryptic-plasmid-based shuttle vectors, pLAE001 and pLAE003, were constructed for the PEG-mediated protoplast transformation and the intergeneric conjugation, respectively (Fig. 3) (Hamano et al. 2005). The plasmid pLAE001was constructed with pBBH4 and pNEB193 (commercially available plasmid vector for *E. coli*). The plasmid pLAE003 was constructed with pBBH4 and pK18mob.

In addition, a pNO33 curing strain of *S. albulus* NBRC14147, designated *S. albulus* CR1, was constructed and used as a host strain for the plasmids pLAE001 and pLAE003 (Hamano et al. 2005).

2.3 *PEG-Mediated Transformation of* S. albulus *CR1 Protoplast with pLAE001*

In PEG-mediated protoplast transformation, a transformation frequency of 10^2 transformants per 1 μg DNA was observed using pLAE001, which was prepared

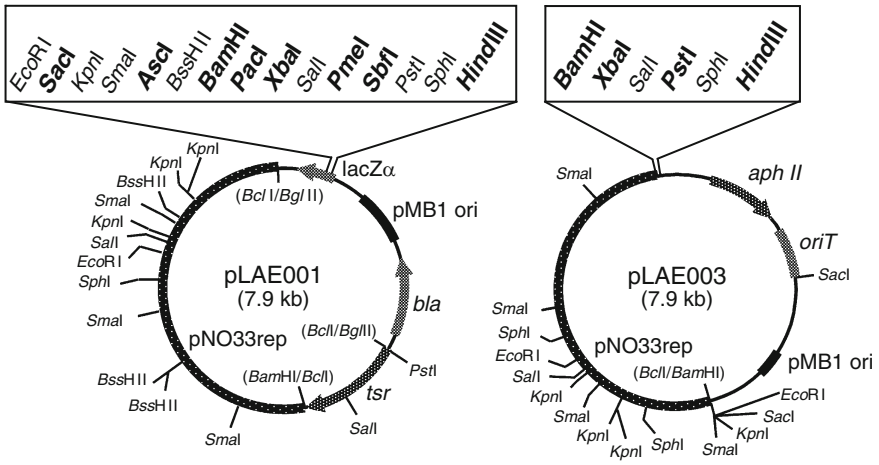

Fig. 3 pNO33-based shuttle vectors. pLAE001 and pLAE003 were used for the PEG-mediated protoplast transformation and intergeneric conjugation from *E. coli* to *S. albulus* CR1, respectively. Bold letters indicate cloning sites for restriction enzymes. Abbreviations: pNO33rep, pNO33 replicon (*Bcl*I–*Bam*HI 4.1 kb); *bla*, β-lactamase gene (Ampicillin resistance gene); *aph II*, aminoglycosides phosphotransferase II (neomycin/kanamycin resistance gene); *tsr*, thiostrepton resistance gene

from *S. lividans* TK23 (Hamano et al. 2005). When pLAE001 prepared from *S. albulus* CR1 was used for the transformation, the efficiency increased 100-fold, demonstrating that *S. albulus* CR1 shows a strong restriction barrier. In fact, the authors reported that pLAE001 prepared from a methylation-proficient *E. coli* strain, such as XL1-blue MRF′, cannot transform *S. albulus* CR1.

2.4 *Conjugal Transfer of the* oriT*-Vector, pLAE003, from* E. coli *to* S. albulus *CR1*

Recently, there has been considerable interest in the use of intergeneric conjugation as a means of gene transfer to bypass protoplast formation and regeneration (Mazodier et al. 1989; Matsushima et al. 1994; Flett et al. 1997; Voeykova et al. 1998; Paranthaman and Dharmalingam 2003; Choi et al. 2004; Stinchi et al. 2003). In *S. albulus* CR1, exconjugants were obtained employing a standard procedure, although the conjugation efficiency was very low (exconjugants per recipient spores: 9.0×10^{-8}). Nihira et al. have reported that the optimal concentration of $MgCl_2$ for conjugation differs among the various strains (Choi et al. 2004). In fact, in *S. albulus* CR1, the solid medium containing an optimal concentration of $MgCl_2$ (40 mM) provided the highest frequency (4.0×10^{-7}) (Hamano et al. 2005).

2.5 *Construction of a Genetically Engineered Strain of* S. albulus *CR1 for ε-PL Overproduction*

Shima et al. have reported that the L-lysine molecule is directly utilized in ε-PL biosynthesis (Shima et al. 1983). In most bacteria, L-lysine is biosynthesized by the amino-acid biosynthetic pathway from L-aspartic acid (aspartate pathway, Fig. 4). This pathway is also involved in the formation of other amino acids (i.e., L-methionine, L-isoleucine, and L-threonine). The first two enzymes in this pathway are aspartokinase (Ask) (EC.2.7.2.4), which catalyzes the phosphorylation of L-aspartic acid to produce L-4-phospho aspartic acid, and aspartate semialdehyde dehydrogenase (Asd) (EC.1.2.1.11), which reduces L-4-phospho aspartic acid into L-aspartate 4-semialdehyde. These two key enzymes are subject to complex regulation by the end-product amino acids. Because of the complexity of this pathway, different bacterial species have evolved diverse patterns of Ask regulation. For example, *E. coli* and *Bacillus subtilis* have three separate Ask isozymes, each controlled by one of the end products of the aspartate pathways, diaminopimelic acid, lysine, threonine, and methionine (Hitchcock et al. 1980; Theze et al. 1974; Zhang et al. 1990; Zhang and Paulus 1990). In contrast, only one Ask has been described in

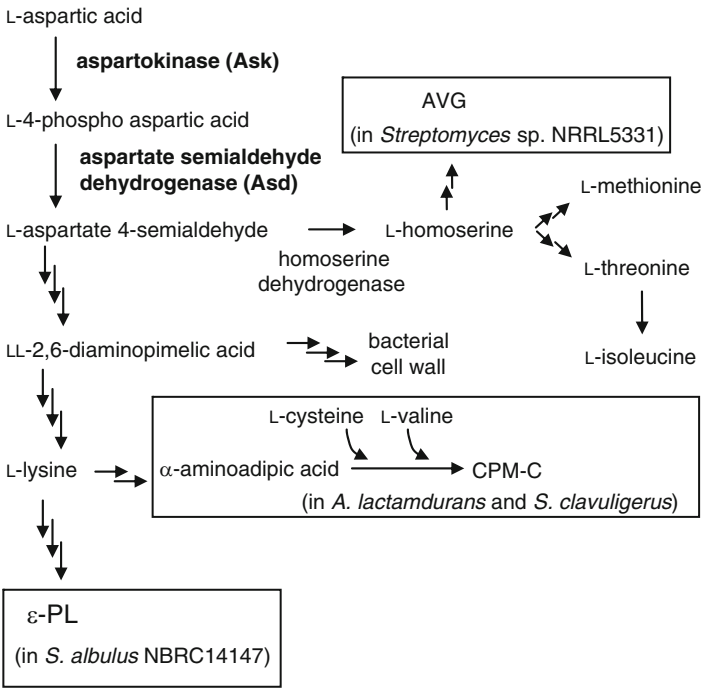

Fig. 4 The amino-acid biosynthetic pathway from L-aspartic acid (aspartate pathway). *AVG* aminoethoxyvinylglycine; *CPM-C* cephamycin C

antibiotic-producing actinomycetes. There are some reports of cloned *ask* genes from the rifamycin SV producer *Amycolatopsis mediterranei* (Zhang et al. 1999), from the cephamycin C (CPM-C, Fig. 4) producers *Amycolatopsis lactamdurans* (Hernando-Rico et al. 2001) and *Streptomyces clavuligerus* (Tunca et al. 2004), and from the aminoethoxyvinylglycine (AVG, Fig. 4) producer *Streptomyces* sp. NRRL5331 (Cuadrado et al. 2004). These studies have shown that regulatory mechanisms can differ even among the actinomycetes. Zhang et al. have reported inhibition of "*A. mediterranei*" Ask by L-lysine alone (Zhang et al. 2000), whereas Asks of *A. lactamdurans* and *S. clavuligerus* are feedback-regulated by the concerted action of L-lysine and L-threonine (Tunca et al. 2004; Hernando-Rico et al. 2001). Interestingly, these experimental observations indicate that the Asks of *A. lactamdurans* and *S. clavuligerus* (CPM-C producers) are slightly more resistant to concerted feedback-inhibition than Ask IIIs of *E. coli* (Ogawa-Miyata et al. 2001) and *B. subtilis* (Kobashi et al. 2001), although there is no discussion of this result in the respective reports. As such, the L-lysine productivity of these strains should be higher than those of *E. coli* and *B. subtilis*. This result could be due to the need for that, L-lysine, which is one of biosynthetic precursors of CPM-C, must be fully served to not only nascent protein biosynthesis but also the CPM-C biosynthesis (Fig. 4).

The ε-PL producer, *S. albulus* NBRC14147, can produce a large amount of ε-PL (usually 1–3 g/l). Therefore, Ask(s) of *S. albulus* was also expected to be potentially resistant to feedback-inhibition by L-lysine and/or L-threonine to provide sufficient amounts of L-lysine for ε-PL biosynthesis, similar to those of *A. lactamdurans* and *S. clavuligerus*. In fact, Hamano et al. demonstrated that the recombinant Ask of *S. albulus* NBRC14147 was found to be partially resistant to feedback-inhibition *in vitro* analysis (Hamano et al. 2007).

Hernando-Rico et al. have reported a construction of the feedback-resistant Ask of *A. lactamdurans* by substitutions of the two amino-acid residues, Ser^{301} and Gly^{345} (Hernando-Rico et al. 2001). However, a homologous expression of the mutated Ask has not been carried out due to the extreme difficulty in transforming *A. lactamdurans*. In *S. albulus* CR1, by random mutagenesis of the *ask* gene with error-prone PCR and the subsequent site-directed mutagenesis, Hamano et al. successfully constructed the mutated Ask, Ask (M68V), whose feedback-inhibition regulation was completely removed. Ask of *S. albulus* CR1 also has Ser^{301} and Gly^{345}, but no mutated Ask with substitutions of these two amino-acid residues were obtained. Rather, rAsk (M68V) was more appropriate for homologous expression in *S. albulus* CR1 to investigate whether L-lysine accumulation in the cell leads to ε-PL overproduction, as the calculated V_{max}^{Asp}/K_m^{Asp} values of rAsk (M68V) were ten-fold higher than that of the rAsk (wild type).

Using the genetic system for *S. albulus* CR1 described earlier, Hamano et al. constructed the genetically engineered strain of *S. albulus* CR1, which produced rAsk (M68V). Compared with the productivity in the use of Ask (wild), the homologous expression of Ask (M68V) predictably conferred a higher productivity of ε-PL (Fig. 5) (Hamano et al. 2007).

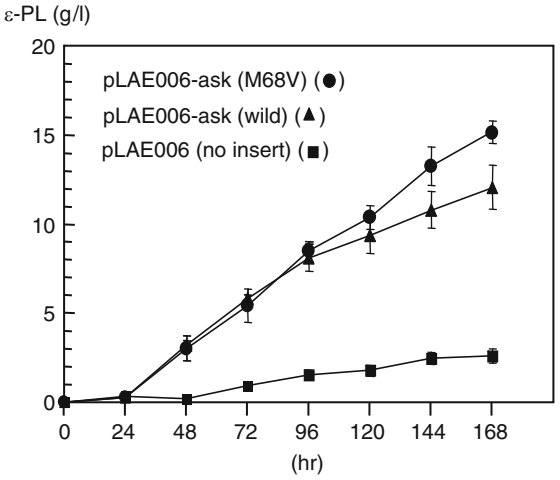

Fig. 5 The ε-PL productivities in the *S. albulus* CR1 strains expressing Ask (wild) or Ask (M68V). The *S. albulus* CR1 strains harboring pLAE006 (pLAE003 derivative harboring the *erm*E* promotor), pLAE006-ask (wild), and pLAE006-ask (M68V) were cultivated in ε-PL production medium containing 100 μg/ml neomycin by using a 5-l capacity bench scale jar-fermentor

3 ε-PL Synthetase

Two amino-acid "homopolymers" comprising a single type of amino acid are known in nature (Oppermann Sanio and Steinbuchel 2002): γ-poly-glutamic acid (γ-PGA) and ε-PL. The latter, which consists of 25–35 L-lysine residues with linkages between α-carboxyl groups and ε-amino groups (Fig. 1), exhibits antimicrobial activity against a spectrum of microorganisms including bacteria and fungi (Oppermann Sanio and Steinbuchel 2002; Shima et al. 1982; Shima et al. 1984). Due to its safety and biodegradability, it is used as a food preservative in several countries. The biological activity of ε-PL is known to be dependent on its molecular size. Shima and coworkers investigated the relationship between the molecular size of ε-PL and its antimicrobial activity against *E. coli* K-12 (Shima et al. 1984). ε-PL with more than nine L-lysine residues severely inhibited microbial growth; however, the L-lysine octamer demonstrated negligible antimicrobial activity. In contrast, chemically synthesized α-poly-L-lysine that contains a considerably longer chain of L-lysine residues (50 residues), which show linkages between the α-carboxyl and α-amino groups, demonstrates a lower activity than ε-PL. Thus, polymerization of L-lysine via an isopeptide bond is required to exert its biological activity, and the polymerization mechanisms involved in the chain-length diversity of ε-PL are of particular interest.

Nonribosomal peptide synthetases (NRPSs) are multifunctional enzymes consisting of semiautonomous domains that synthesize numerous secondary metabolites (Walsh 2003; Schwarzer et al. 2003; Mootz et al. 2002; Marahiel et al. 1997). Using an assembly-line logic comprising multiple modules, they utilize a thiotemplated mechanism to activate, tether, and modify amino-acid monomers, sequentially elongating the peptide chain before releasing the complete peptide. The order and number of modules of a NRPS system determine the sequence and length of the

peptide product. It has been reported that ε-PL might be produced by NRPSs using a thiotemplate mechanism (Kawai et al. 2003; Saimura et al. 2008). However, as they used the crude extract of an ε-PL-producing microorganism, it was not possible to confirm the biosynthetic mechanism. Additionally, the chain-length diversity of ε-PL products is difficult to explain with this generic model. In addition, Hamano et al. recently identified and characterized ε-PL-degrading enzymes in an ε-PL producer, *S. albulus* NBRC14147 (Hamano et al. 2006). This raised the question of whether the degrading enzymes, rather than the biosynthetic machinery, might be responsible for generating products of diverse chain length. Actually, this section will reveal that the chain-length diversity of ε-PL is directly generated by the synthetase.

3.1 Purification of Pls from S. albulus *NBRC14147*

S. albulus NBRC14147 was grown to the late logarithmic phase, in which ε-PL production was observed. The mycelium collected from a 700 ml-culture broth were sonicated and centrifuged to obtain a cell-free extract. After ultracentrifugation of the cell-free extract, ε-PL-synthesis was observed in an insoluble fraction, suggesting that Pls is an insoluble protein such as a membrane protein. Yamanaka et al. therefore solubilized this fraction with a nonionic detergent, Nonidet P-40 (NP-40) (Yamanaka et al. 2008). By successive purification steps including column chromatography, the solubilized Pls was finally purified 168-fold to apparent homogeneity. The relative molecular mass of the native enzyme as estimated by gel-filtration chromatography was 270 kDa. However, the molecular mass as estimated by denaturing SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was 130 kDa, suggesting that Pls is a homodimer.

3.2 Enzymatic Characterization of the Purified Pls

The purified enzyme was incubated with L-lysine and ATP. A polydisperse group of enzyme-dependent polymer products were detected, with their retention times corresponding to those of reference standard polymers consisting of 3–17 residues (Fig. 6). Incubation of Pls with L-lysine for different times did not change the relative amounts of each polymer produced during the polymerization reaction. ESI-tandem MS (ESI-MS/MS) analysis of the enzymatically synthesized polymer with 14 residues was identical to that of the reference standard polymer with 14 residues. Identical mass spectra to the reference standards were also observed for products of other chain lengths. Chemical modification of the synthesized polymers using 2,4-dinitrophenol (DNP) followed by TLC analysis against reference DNP-modified α- and ε-lysine polymers demonstrated that the α-amino groups of the L-lysine residues were labeled with DNP. This result shows that the polymer

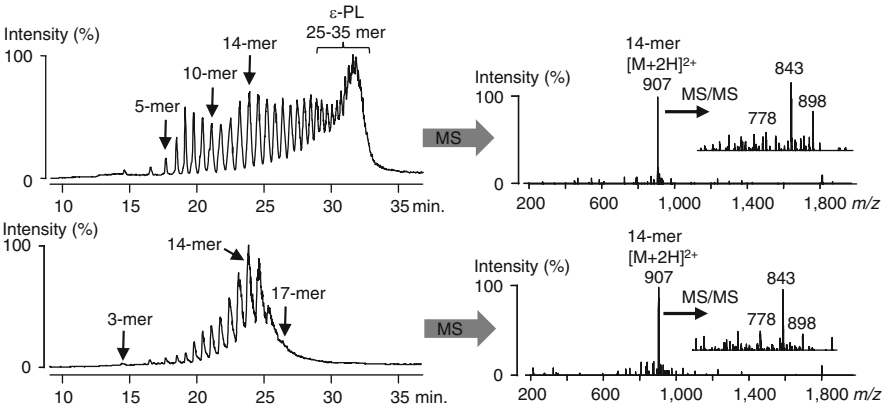

Fig. 6 Identification of the Pls reaction products. The reaction mixture (lower chromatogram and ESI mass spectrum) and a hydrolysate of ε-PL (upper chromatogram and ESI mass spectrum) were analyzed by HPLC/ESI-MS. The ε-PL hydrolysates (0.1 mg/ml) were prepared by hydrolysis with 1N HCl

consists of L-lysine residues with linkages between the α-carboxyl and ε-amino groups and confirms that the polydisperse polymer products are indeed ε-PL.

Yamanaka et al. investigated other enzymatic properties (Yamanaka et al. 2008). The enzyme required Mg^{2+}, ATP, 20–30% glycerol, 2 mM dithiothreitol, and 0.2–0.4% NP-40 for full activity. ATP was converted to AMP during the Pls reaction. No activity was detected with other nucleotides such as GTP, CTP, and TTP in the Pls polymerization reaction. Maximum activity occurs at an optimum pH of 8.5. The effect of temperature on the enzyme activity was investigated over the range of 10–45°C, with the maximum activity being observed at 25–30°C.

3.3 Cloning of the Gene Encoding Pls

To clone the gene encoding Pls, some of the amino-acid sequences of Pls were determined, and then PCR primers are designed. Using the PCR product as a probe, a 33-kbp DNA fragment containing the *pls* gene from *S. albulus* NBRC14147 was obtained. Interestingly, a BLAST database search showed that homologous genes are widely distributed among microorganisms. The *pls* gene encoded a protein of 1,319 amino acids containing the internal amino-acid sequences previously determined. The calculated molecular mass (138,385 Da) was in good agreement with the result from the SDS–PAGE analysis of the purified Pls. The authors also constructed a knockout mutant of the putative *pls* gene using the genetic system described earlier in Sect. 3. The mutant produced no ε-PL, demonstrating that the cloned gene encodes Pls (Yamanaka et al. 2008).

With the protein sequence in hand, they determined the predicted function of the enzyme. In a traditional NRPS, the amino-acid substrate is activated as an

acyl-*O*-AMP by an adenylation domain (A-domain) and subsequently loaded onto the 4′-phosphopantetheine (4′-PP) arm of the adjacent thiolation domain (T-domain) (Walsh 2003; Schwarzer et al. 2003; Mootz et al. 2002; Marahiel et al. 1997), resulting in the formation of an acylthioester and AMP release. A domain search showed the presence of an A-domain and a T-domain in the N-terminal region of the Pls. Their prior observation that AMP is released during the course of the reaction, along with the similarity of the ten residue sequence that confers substrate specificity in the putative Pls A-domain to that of the A-domain of the BacB (Challis et al. 2000) protein that adenylates L-lysine and the presence of Ser553 from the putative T-domain in proper alignment to be a 4′-PP-binding residue as compared with the T-domains of traditional NRPSs provides strong support for the classification of these regions of Pls as A- and T-domains. This distinguishes the mechanism of ε-PL biosynthesis from that of γ-PGA (Oppermann Sanio and Steinbuchel 2002), glutathione (Hibi et al. 2004), or cyanophycin (Oppermann Sanio and Steinbuchel 2002), which require phosphorylation of the carboxyl group.

Surprisingly, given the evidence for the presence of the A- and T-domains, Pls had no domain with significant sequence similarity to the traditional condensation domains (C-domains) that are crucial in peptide bond formation in NRPSs. Furthermore, it had no traditional thioesterase domain (TE-domain), which catalyzes release of the final product from NRPS enzymes by hydrolysis to the free acid or cyclization to an amide or ester. Instead, a physicochemical analysis of the Pls amino-acid sequence with SOSUI (Hirokawa et al. 1998) suggested the existence of six transmembrane domains (TM-domains) surrounding three tandem soluble domains that display significant sequence similarity (with pairwise identities of 27, 22, and 23%) (Fig. 7). Alignment of the tandem domains with traditional C-domains demonstrated that the tandem domains did contain motifs showing similarity to His-motifs, or the HHxxxDG sequences found in all traditional C-domains, but the two histidine residues, known to be critical for catalysis, were not conserved. However, both the primary sequence and predicted three-dimensional structure of these domains showed similarity to acetyltransferases, which do show structural similarity to C-domains (Bergendahl et al. 2002; Keating et al. 2002). For this reason, Yamanaka et al. named the tandem sequences the C1-, C2-, and C3-domains, with the expectation that they would have a role in peptide bond formation (Yamanaka et al. 2008).

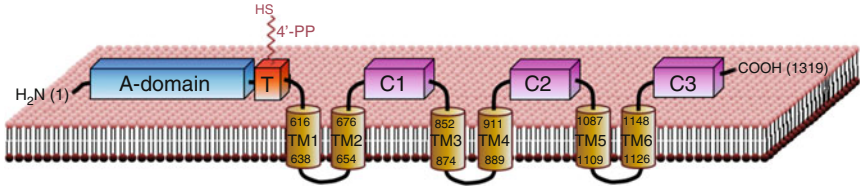

Fig. 7 Domain architecture of Pls. The A-, T-, six TM-domains, and three tandem domains (C1-, C2-, and C3-domain) are shown schematically. The numbers on Pls are the amino-acid residue numbers

3.4 Catalytic Mechanism of Pls

Yamanaka et al. explored the catalytic mechanism of the Pls using an ATP–PP_i exchange assay (Yamanaka et al. 2008). They observed Pls-mediated adenylation of L-lysine, but not any other proteinogenic amino acid (Fig. 8a). To investigate the function of the putative T-domain, they constructed a His-tagged recombinant Pls containing only the A- and T-domain (rPls-AT) using *S. lividans* TK23 as a heterologous host. Incubation of rPls-AT with L-[U-^{14}C]lysine and ATP as substrates resulted

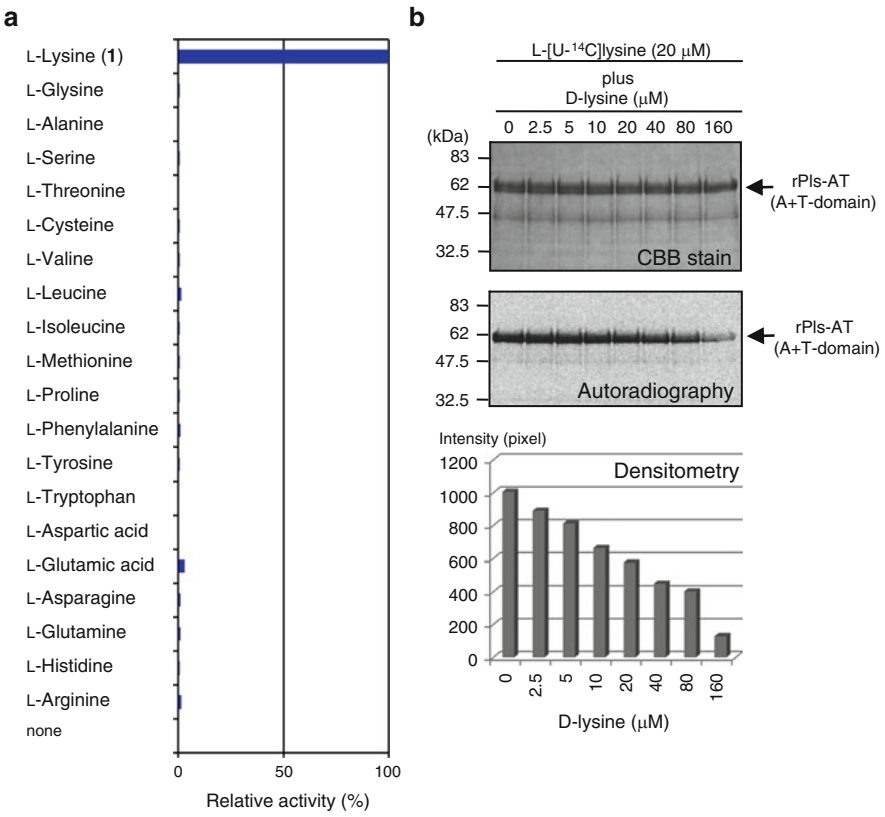

Fig. 8 Functional analysis of Pls. (**a**) The relative adenylation activities were determined on proteinogenic amino acids. Each value is represented as the mean of three experiments. (**b**) Aminoacylation of rPls-AT with L-[U-^{14}C]lysine was investigated. rPls-AT (Fig. 9) was incubated with L-[U-^{14}C]lysine plus D-lysine at 30°C for 12 h. The reaction mixtures were then subjected to SDS–PAGE (4 μg protein per lane). Proteins were stained with CBB R-250. The dried gel was exposed on an imaging plate and visualized by BAS-2500. The data from densitometry analysis of the autoradiography was shown. Data from this experiment is consistent with the fact that rPls-AT was modified with the 4′-PP cofactor in *S. lividans* TK23; the 4′-PP was posttranslationally transferred from Coenzyme A to the conserved serine residue (Ser553) of the T-domain by endogenous 4′-phosphopantetheinyl transferase(s), thus converting the inactive apo-form to its active holo-form

in loading of the labeled lysine onto the enzyme (Fig. 8b). However, ε-PL production was not detected, suggesting that the three tandem domains (C1-, C2-, and C3-domain) of Pls are essential for catalyzing the L-lysine polymerization reaction. To gain a better understanding of the function of the tandem domains, two additional recombinant Pls enzymes, rPls-ATC1C2 (lacking the C3-domain) and rPls-ATC1 (lacking both the C2- and C3-domain), were constructed. As observed for rPls-AT, which lacks all three C domains, no polymer products were detected for rPls-ATC1C2 and rPls-ATC1, suggesting that either the C3-domain or the interconnected action of all three domains is essential for peptide-bond formation.

As described earlier, Pls is predicted not to have a TE-domain, which is traditionally required for release of the product from the NRPS machinery. This suggested that the growing polymer products are not covalently attached to Pls during the polymerization reaction. To investigate this hypothesis, Pls was incubated with L-[U-^{14}C] lysine and ATP. Analysis of the reaction mixture confirmed that Pls was radiolabeled during the reaction (Fig. 9a); however, performic acid treatment of the labeled Pls, which will release any small molecules attached via thioester bonds, only returned L-[U-^{14}C]lysine monomers instead of any polylysine chains (Fig. 9b). These results strongly suggested that the growing polymer products are not covalently attached to Pls during the polymerization reaction. Yamanaka et al. similarly observed that short-chain ε-PL oligomers (3-mer to 9-mer) are neither adenylated nor incorporated into polymers primed with free, deuterated L-lysine (L-Lys-d8); instead, Pls produced L-Lys-d8 homopolymers. To further test this mechanism, they employed L-lysine ethyl and methyl esters as substrates. HPLC/ESI-MS analysis of the reaction revealed that Pls produced the corresponding ethyl (Fig. 10) and methyl ester forms of ε-PL. While retention of the final ester functionality confirmed that the growing polymers or polymer products are not tethered to Pls via covalent bonds such as thioesters or esters during the polymerization reaction (which would result in complete loss of the ester group), it also raised the question of how the enzyme catalyzes polymerization using ester substrates at all. The answer lay in a unique function of Pls: the A-domain converts these L-lysine esters to L-lysine by its own esterase activity (Fig. 11), then the resulting L-lysine can be adenylated and loaded to the T-domain.

3.5 Substrate Specificity of Pls

Given that Pls can accept lysine esters as substrates, the substrate specificity of Pls was investigated further with seven L-lysine analogs (Fig. 12). L-Ornithine (L-Orn), L-kynurenine (L-KNR), and 3-amino-L-tyrosine (L-ATY) were not accepted as substrates. Pls was able to adenylate D-lysine (relative activity = 19%), but AMP-forming activity was not detected, suggesting that the adenylated D-lysine is not loaded onto the T-domain. Indeed, D-lysine serves as an inhibitor of the aminoacylation of L-[U-^{14}C]lysine (Fig. 8b), in agreement with a model where D-lysine is stalled on the A-domain. Pls did successfully adenylate and pantetheinylate (as monitored by AMP formation) the rest of the analogs, including (5R)-5-hydroxy-L-lysine

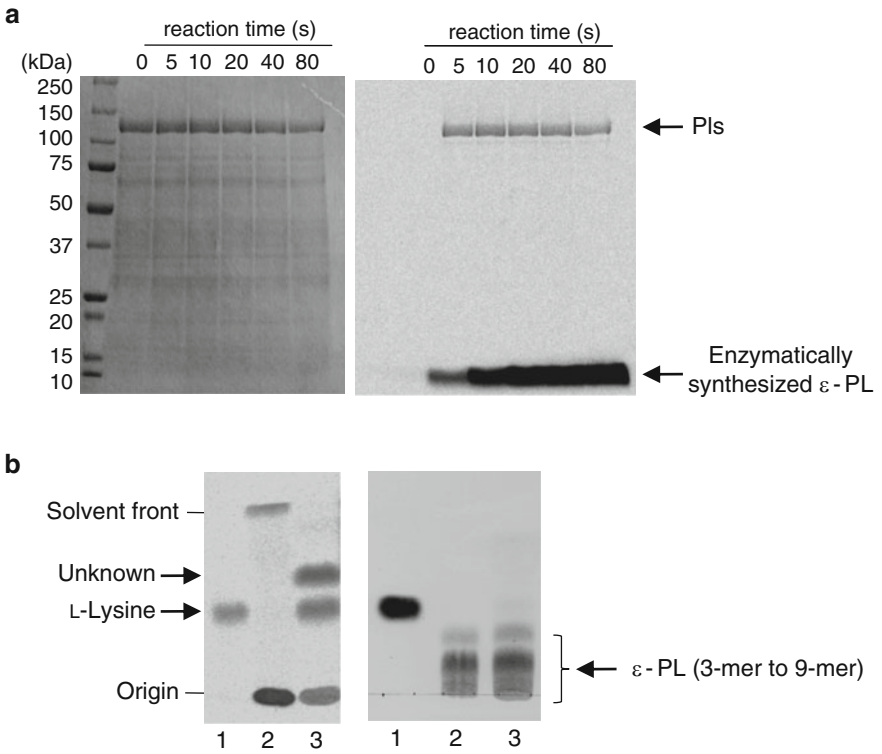

Fig. 9 Performic-acid oxidation of the radiolabeled Pls formed in the Pls reaction with L-[U-^{14}C]lysine. (**a**) After incubation of Pls with L-[U-^{14}C]lysine and ATP at 5°C for 0–80 s, the reaction mixtures were subjected to SDS–PAGE (5–20% gradient gel, 16 μg protein per lane). Proteins were stained with CBB R-250 (image on the *left*). The dried gel was exposed on an imaging plate and visualized by BAS-2500 (Fuji film; image on the *right*). (**b**) The reaction mixture incubated for 10 s was further used for the oxidation experiments of Pls. L-[U-^{14}C] lysine (lane 1), and the radiolabeled Pls treated with performic (lane 3) and formic acid (lane 2) were analyzed by silica gel thin-layer chromatography (TLC) (*left* image). The dried TLC was exposed on an imaging plate and visualized by BAS-2500. Additionally, to confirm that these acids do not hydrolyze the short-chain ε-PL oligomers, the oligomers (3-mer to 9-mer) were treated with formic (lane 2) and performic acids (lane3) and analyzed by silica gel TLC (image on the *right*). L-lysine (lane 1) was also used as the reference standards to confirm the mobility of the short-chain ε-PL oligomers. The samples were detected with ninhydrin reagent. In this oxidation experiments, an amino acid bound to enzyme as thioester is released from the enzyme not by formic acid but by performic acid. In the *left* image of panel (**b**), we detected the extra band (lane 3), which is known as the commonly observed unknown spots in performic oxidation experiments

(L-HLY; relative activity for adenylation, 43%; relative activity for AMP-formation, 6%), *O*-(2-aminoethyl)-L-serine (L-AES; corresponding values, 13 and 12%), and *S*-(2-aminoethyl)-L-cysteine (L-AEC; corresponding values, 5 and 6%). However, no homopolymers from these analogs were observed (see the lower chromatograms in Fig. 13b–d). In contrast, when a small amount of L-lysine was added to the reaction

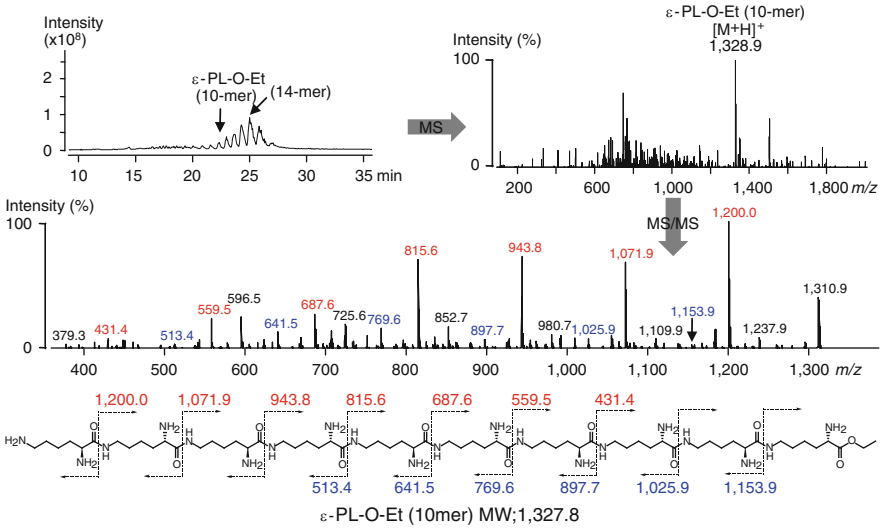

Fig. 10 Pls reaction with L-lysine ethyl ester. The Pls polymerization reaction with 2 mM L-lysine ethyl ester was performed and analyzed by HPLC/ESI-MS. The mass spectra and MS/MS spectra of the selected polymers (10-mer) in the reactions are shown

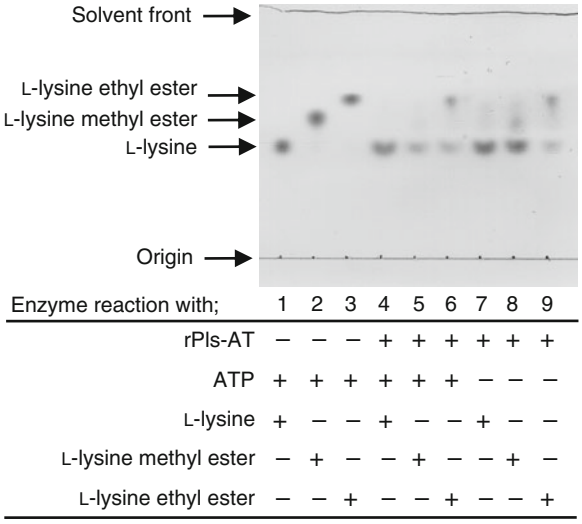

Fig. 11 Esterase activity in the A-domain. The rPls-AT reaction with 2 mM L-lysine, 2 mM L-lysine methyl ester, or 2 mM L-lysine ethyl ester was performed and analyzed by silica gel TLC. TLC was developed in 1-butanol/pyridine/acetic acid/H_2O, 2:1:1:2 (v/v). The products were detected with ninhydrin reagent. These results demonstrated that the esterase activities were not dependent on ATP. Addition, (+); No addition, (−)

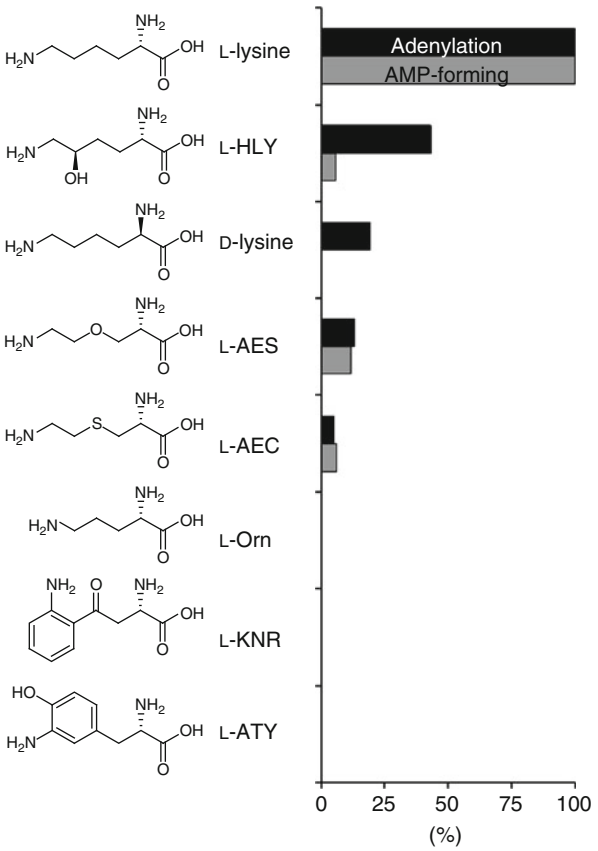

Fig. 12 Substrate specificities in the A- and T-domain of Pls. The relative activities in the adenylation and AMP-forming activities were determined on L-lysine and the L-lysine analogues. Each value is represented as the mean of three experiments. Error bars are not shown, because relative standard deviations of less than 5% were commonly calculated. L-HLY, (5R)-5-hydroxy-L-lysine; L-AES, *O*-(2-aminoethyl)-L-serine; L-AEC, *S*-(2-aminoethyl)-L-cysteine; L-Orn, L-ornithine; L-KNR, L-kynurenine; L-ATY, 3-amino-L-tyrosine

mixtures, heteropolymers consisting of L-lysine and the analogs were produced (see the upper chromatograms in Fig. 13b–d). These results indicate that the A- and T-domains are partially tolerant to substrate analogs, whereas the three tandem acetyltransferase domains show high specificity for L-lysine.

4 Concluding Remarks and Future Perspectives

In the ε-PL producing strain *S. albulus* NBRC14147, Hamano and coworkers successfully developed the genetic system, which was a powerful tool to construct a genetically engineered strain and to investigate the biosynthetic mechanism

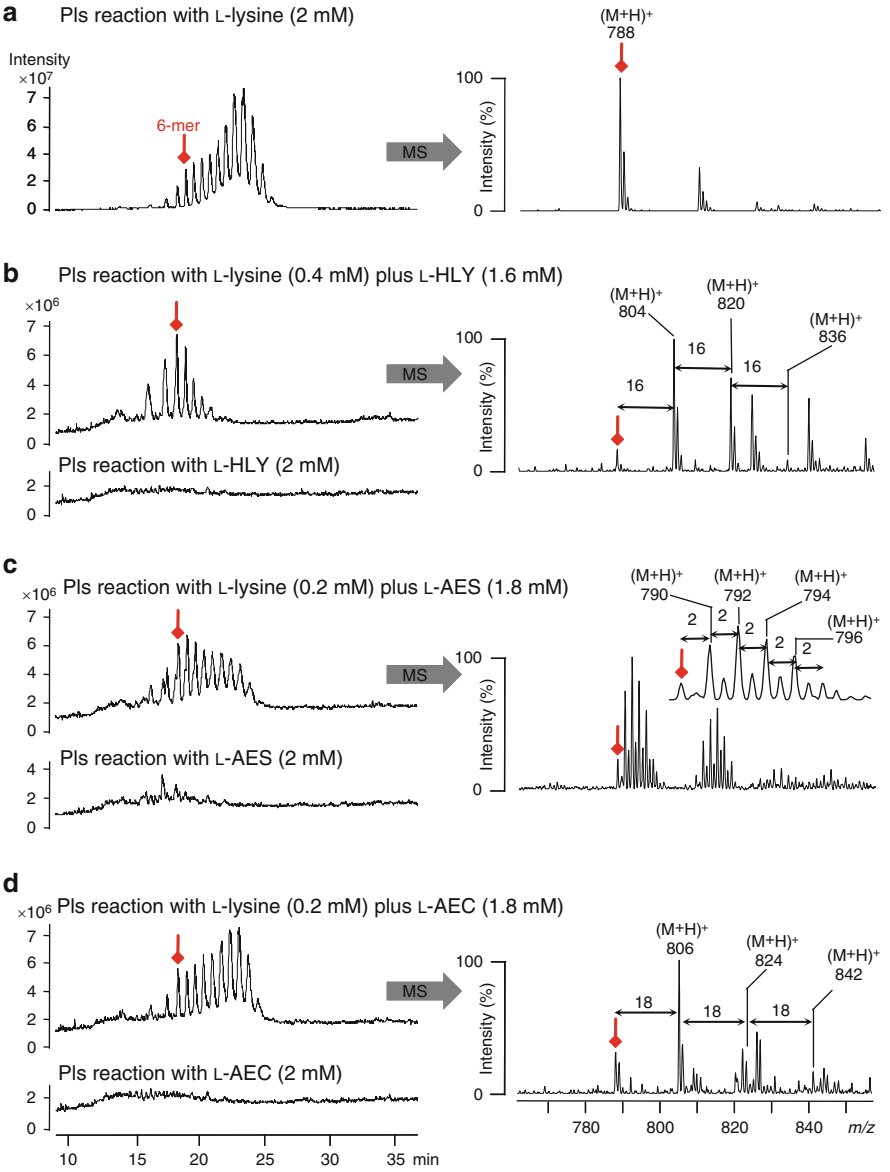

Fig. 13 *In vitro* production of heteropolymers consisting of L-lysine and the L-lysine analogues. The Pls reaction with 2 mM L-lysine (**a**), 1.6 mM L-HLY plus or minus 0.4 mM L-lysine (**b**), 1.8 mM L-AES plus or minus 0.2 mM L-lysine (**c**), or 1.8 mM L-AEC plus or minus 0.2 mM L-lysine (**d**) was performed and analyzed by HPLC/ESI-MS. The mass spectra of the selected polymers (6-mer) in the reactions are shown in the *right-hand* panels

of ε-PL. They also purified a membrane protein with six TM-domains, from the membrane fraction of *S. albulus* NBRC14147, and characterized it as Pls, the biological machinery for ε-PL synthesis. Their explorations of Pls resulted in the development of a new model for the generation of chain-length diversity of ε-PL products, which integrates traditional NRPS logic, in the form of A- and T-domains, with amino-acid ligase functionality in three tandem domains that show similarity to both acetyltransferases and, through these transferases, traditional C-domains. The catalytic mechanism is initiated in N-terminus by the A- and T-domains with the adenylation and transfer of an incoming L-lysine monomer (or "extending unit"), with polymerization occurring as freely diffusible substrates (or "priming units") are added by the C-terminal tandem domains to the extending unit (Fig. 14). Since this cycle has no predetermined endpoint, other than the loss of the noncovalently bound polymer chain to solution, Pls acts iteratively for ε-PL chain growth to obtain a multitude of chain lengths (observed in their studies as ranging from 3 to 17 residues). This mechanism, in which a single polymer is created and then released (Fig. 14), is supported not only by the characterization of more basic Pls properties such as ATP turnover and ε-amine linkage formation but also by the observations that there was no difference in the relative amounts of each polymer during the polymerization reaction and that preexisting short chain polymers could not be incorporated into new chains (i.e., that the short chains were not simply intermediates of the longer chains).

Though ε-PL chain-length diversity can be explained by this mechanism, a detailed description of how the growing polymer interacts with the protein remains a subject for future work. Yamanaka et al. hypothesize that the protein contains a long slender-shaped tunnel, or cavity, that is continuously occupied by a growing polymer during the polymerization reaction. This would explain why the polymerization reaction is specific to the ε-amino groups of the priming units, as the similarly reactive α-amino groups could be buried or otherwise protected from reaction by the Pls catalytic cavity (Fig.14).

In the analysis of the sequence of the three tandem domains, Yamanaka et al. identified a region reminiscent of the known His-motif from traditional C-domains, but lacking the histidine residues thought to be required for catalysis. It has been reported recently that the two histidine residues in the His-motif of VibH are also not critical for catalysis (Keating et al. 2002). As VibH, the functional C-domain of a vibriobactin NRPS, utilizes a similar mechanism to Pls – catalyzing peptide-bond formation between an NRPS-bound substrate, dihydroxybenzoate, and the freely diffusible substrate, norspermidin – these combined results may suggest that the absence or lack of necessity of the two histidine residues may define a secondary motif that can be used to identify C-domains operating via characteristic amino-acid ligase-like mechanisms.

Finally, the investigations demonstrated that the chain-length diversity of ε-PL is directly generated by the synthetase, rather than via the differential degradation of a uniform polymer by ε-PL degrading enzymes. With these findings in hand, it will be interesting to determine whether the activity of these degrading enzymes serves to create a shorter polymer with some defined function or simply regenerates the lysine

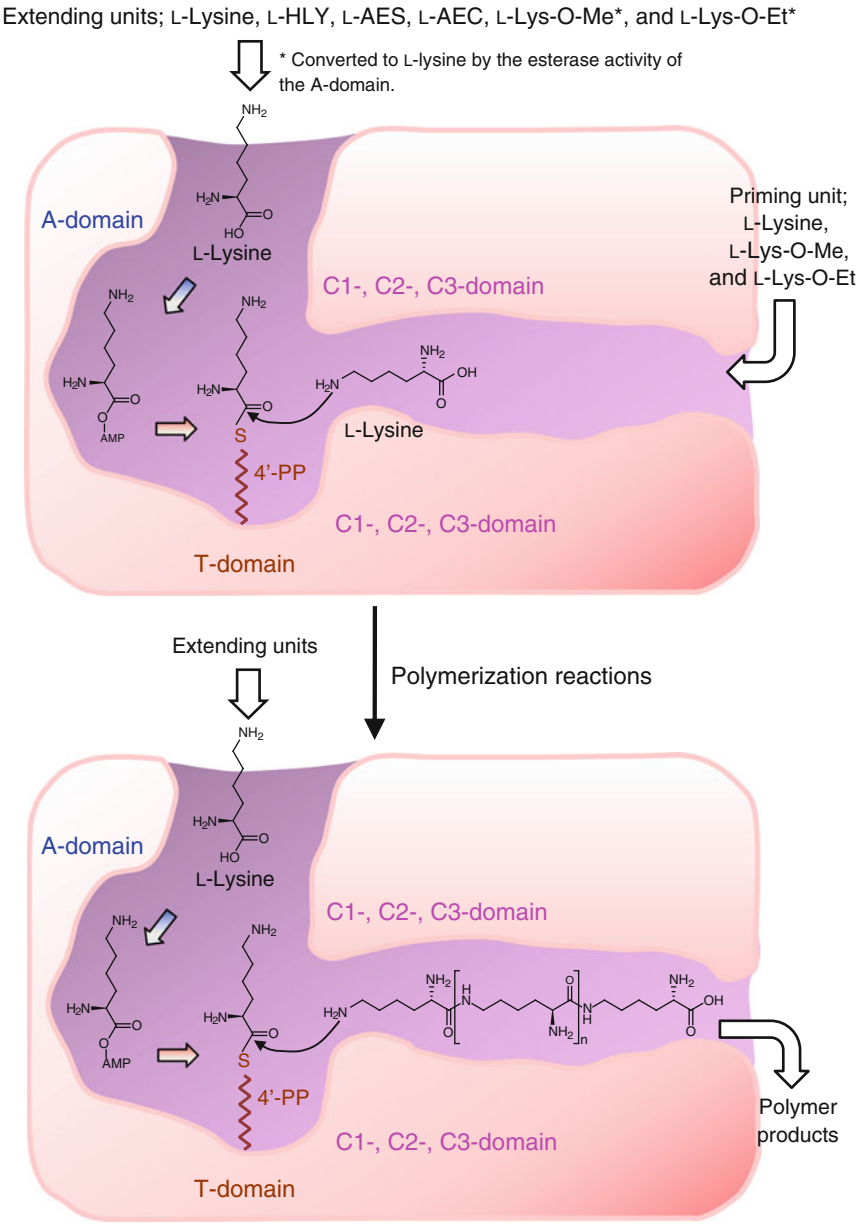

Fig. 14 Pls polymerization mechanism and the proposed model of the Pls catalytic cavity with substrate specificity. The clarified polymerization mechanism is shown schematically. L-Lys-O-Me, L-lysine methyl ester; L-Lys-O-Et, L-lysine ethyl ester

building blocks from the unusual ε-PL chain architecture. Similarly, further explorations of the homologous genes identified in our BLAST search should prove exciting, as their encoded proteins may synthesize amino-acid homopolymers other than ε-PL and γ-PGA. Investigations of these proteins as well as further exploration of Pls should facilitate biosynthetic engineering and help to create new classes of biopolymers.

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Biochemistry and Enzymology of Poly-Epsilon-L-Lysine Degradation

Toyokazu Yoshida

Contents

Abstract Poly-ε-L-lysine (ε-poly-L-lysine, ε-PL) is used as a food additive on the basis of its strong antimicrobial activity (Fine chem 29:18–25, 2000). ε-PL is industrially produced by fermentation process using *Streptomyces albulus*. Recently, the biosynthetic mechanism (Nat Chem Biol 4:766–772, 2008) and microbial degradation of ε-PL (FEMS Microbiol Lett 207:147–151, 2002; Arch Microbiol 178:325–330, 2002; J Biosci Bioeng 96:92–94, 2003; Appl Microbiol Biotechnol 72:173–181, 2006) have been reported. The ε-PL-degrading activity is detected in ε-PL-tolerant bacteria, indicating the contribution of ε-PL-degrading activity to bacterial growth in the presence of ε-PL. The purified ε-PL-degrading enzymes catalyze the endo- and/or exo-type degradation of ε-PL (FEMS Microbiol Lett 207:147–151, 2002; J Biosci Bioeng 96:92–94, 2003). Various peptides and proteins serve as substrates for the ε-PL-degrading enzymes. Probably, the adventitious possession of proteases, exhibiting high ε-PL-degrading activity, enables ε-PL-tolerant bacteria to grow well even in the presence of ε-PL. ε-PL-producing *Streptomyces* strains also possess high ε-PL-degrading activity in their membrane fraction (Arch Microbiol 178:325–330, 2002). ε-PL-degrading enzyme

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purified from *S. albulus* is characterized as a zinc-containing aminopeptidase. In *Streptomyces* strains, the correlative distribution of ε-PL-degrading activity and ε-PL-producing activity is observed, suggesting that the ε-PL-degrading activity is localized in the cell membrane of ε-PL producers for self-protection against ε-PL.

1 Introduction

Since the discovery of poly-ε-L-lysine (ε-poly-L-lysine, ε-PL) in the 1970s (Shima and Sakai 1977), many investigators have noted its wide spectrum of antimicrobial activity, and ε-PL has been widely used as a food additive (Hiraki 2000). ε-PL can be produced industrially, and the studies on its further applications are continuing. ε-PL is a homo-poly amino acid characterized by the peptide bond between α-carboxyl group and ε-amino group. Peptide bonds of protein and peptide are hydrolyzed by various enzymatic reactions. However, there have been few reports on the biological degradation of ε-PL in natural environment or in vivo. Although the safety of ε-PL as a food additive was demonstrated experimentally using rats (Neda et al. 1999), the biological decomposition or utilization of ε-PL in rats was not studied. ε-PL-degrading microorganisms have been isolated from natural environment as ε-PL-tolerant bacteria (Kito et al. 2002a, 2003), in spite of high antimicrobial activity of ε-PL for various microorganisms (Hiraki 2000). Enzymological characterization of the ε-PL-degrading enzymes from ε-PL-tolerant bacteria reveals that the degrading activity of ε-PL-tolerant bacteria is not specialized for ε-PL degradation. ε-PL-degrading activity is also detected in ε-PL-producing *Streptomyces albulus* cells (Shima et al. 1983; Hiraki et al. 1998; Kahara et al. 2001). The presence of ε-PL-degrading enzyme is disadvantageous for industrial ε-PL production by *S. albulus*. However, the enzyme catalyzing ε-PL degradation in *S. albulus* had not been verified, until Kito et al. (2002b) found the activity in the membrane fraction of the ε-PL producer. The recent functional analysis of the *pld* gene encoding ε-PL-degrading enzyme of *S. albulus* (Hamano et al. 2006) demonstrated that *S. albulus* probably possesses, at least, two kinds of ε-PL-degrading enzymes.

2 Assay for ε-PL-Degrading Activity

In this chapter, "ε-PL" indicates a mixture of polymers with 25–35 L-lysine residues. Studies on ε-PL have been mainly carried out using the preparation produced by *S. albulus*, the polymerization degree (*n*) being 25–35.

ε-PL consists of 25–35 L-lysine residues linked with the α-carboxyl and ε-amino groups, and the mode of ε-PL degradation is divided into exo-type and endo-type. Therefore, the degradation activity for ε-PL is generally calculated from residual ε-PL concentration in culture medium and reaction mixture. The best simple and

rapid method to determine ε-PL concentration is a colorimetric procedure using an anionic dye, methyl orange (Itzhaki 1972). In this method, ε-PL interacts with methyl orange on the basis of its cationic property and forms a water-insoluble complex. ε-PL concentration can be estimated from the absorbance at 465 nm of the methyl orange remaining in solution. Free L-lysine and short lengths of L-lysine polymers do not associate with methyl orange, and so the colorimetric method can estimate the total amount of long lengths of L-lysine polymers.

The polymerization degree of L-lysine polymers can be measured using high-performance liquid chromatography (HPLC) (Kito et al. 2002a). Using a reverse-phase column for HPLC analysis, various lengths of L-lysine polymers are individually detected at 215 nm by the gradient elution of acetonitrile in phosphate buffer (pH 2.6) containing $NaClO_4$ and sodium octane sulfonate as an ion-pair reagent. Although the HPLC analysis is time-consuming, the estimation of polymerization degrees of L-lysine polymers is essential to the studies on ε-PL degradation. Free L-lysine concentration released from ε-PL is generally determined using amino acid analyzer or enzymatically using L-lysine-α-oxidase (Kusakabe et al. 1980).

To verify the mode of ε-PL degradation, the polymerization degree and concentration of L-lysine polymers should be analyzed. In the representative time course of exo-type ε-PL degradation, various lengths of L-lysine polymers are constantly detected by HPLC analysis until complete degradation of ε-PL (Fig. 1a), and the sizes of L-lysine polymers are gradually reduced in accordance with the formation of free L-lysine. In the reaction mixture, L-lysine concentration enhances in proportion to time. Therefore, as for the exo-type ε-PL degradation, the degrading activity for ε-PL can be also evaluated as the formed L-lysine concentration. In endo-type ε-PL degradation (Fig. 1b), various lengths of L-lysine polymers are initially

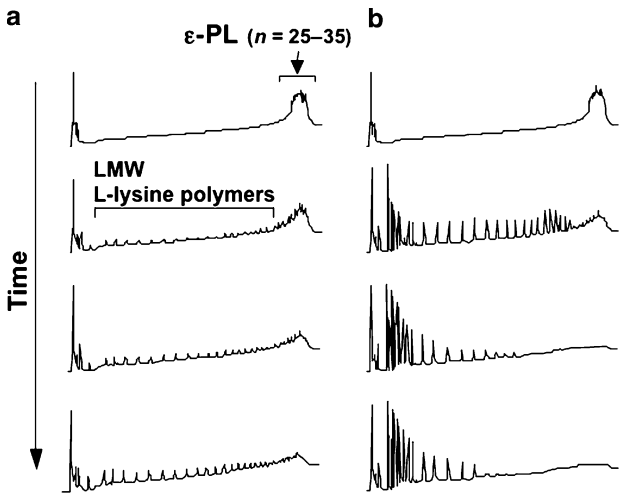

Fig. 1 Analysis of ε-PL degradation using HPLC system. (**a**) exo-type ε-PL degradation; (**b**) endo-type ε-PL degradation. *LMW* low molecular weight

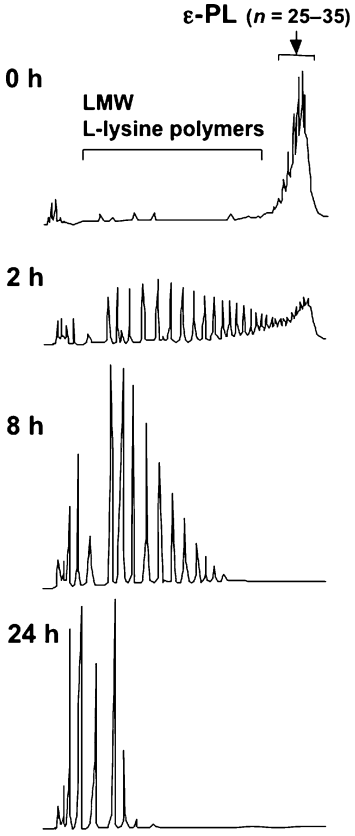

Fig. 2 ε-PL degradation by the purified ε-PL-degrading enzyme from *Chryseobacterium* sp. OJ7. ε-PL was incubated with the purified ε-PL-degrading enzyme for the indicated time, and the polymerization degree of L-lysine was analyzed by HPLC

detected as in the case of exo-type degradation. However, further incubation results in the accumulation of shorter lengths of L-lysine polymers in the reaction mixture. In the case of mixed-type degradation as observed using *Chryseobacterium* sp. OJ7 (Kito et al. 2003), various peptides containing L-lysine residue(s) should be tested as the substrate to confirm the mode of ε-PL degradation (Fig. 2).

For the assay of ε-PL-degrading enzyme in the purification procedures, L-lysyl-*p*-nitroanilide serves as a convenient substrate, irrespective of the mode of ε-PL degradation (Kito et al. 2002a, b, 2003). ε-PL-degrading enzymes efficiently catalyze the hydrolysis of L-lysyl-*p*-nitroanilide; the formed *p*-nitroaniline is easily measured spectrophotometrically with a high sensitivity.

3 ε-PL Degradation by Microorganisms

The notable antimicrobial activity of ε-PL against various microorganisms attracts a great deal of attention for its use as a food preservative. When the amino groups of ε-PL are partially amidated using aromatic carboxylic acids such as 4-chlorobenzoic

acid, the antimicrobial activity of ε-PL is remarkably diminished (Shima et al. 1984). The proposed mechanism of the inhibitory effect of ε-PL on microbial growth is electrostatic adsorption of ε-PL to the cell surface of microorganisms. This is based on the molecule's cationic properties, and leads to stripping of the outer membrane and abnormal distribution of cytoplasm as observed by electron microscopy (Shima et al. 1984). However, the degradation activity for ε-PL has been found in several bacteria isolated from soils as ε-PL-tolerant microorganisms (Kito et al. 2002a, 2003). The tolerance of microorganisms against ε-PL seems to be derived from their ability to degrade ε-PL efficiently. Similarly, ε-PL-producing *Streptomyces* strains possess ε-PL-degrading activity in their membrane fraction, probably, for self-protection against ε-PL.

3.1 ε-PL Degradation by ε-PL-Tolerant Microorganisms

The physiological role of ε-PL for ε-PL-producing *S. albulus* remains unclear. However, ε-PL exhibits antimicrobial activity with a wide spectrum of microorganisms including Gram-positive and Gram-negative bacteria (Shima et al. 1984; Hiraki 2000), and is applied under practical circumstances as a food additive on the basis of its strong antimicrobial activity (Shima et al. 1984; Hiraki 2000); the minimum inhibitory concentration (MIC) of ε-PL for the growth of many bacteria is indicated as below 100 μg ml^{-1} (Shima et al. 1984; Hiraki 2000). Although MICs for fungi are high, the inhibitory effect of ε-PL is superior to that of other antimicrobial compounds used as food preservatives. Kito et al. (2002a, 2003) suspected that ε-PL-tolerant microorganisms might have ε-PL-hydrolyzing proteases. The isolated ε-PL-tolerant bacteria, *Sphigobacterium multivorum* OJ10 (Kito et al. 2002a) and *Chryseobacterium* sp. OJ7 (Kito et al. 2003), exhibited high ε-PL-degrading activity. The enzymes catalyzing ε-PL degradation by both bacteria were purified to homogeneity and characterized.

3.1.1 ε-PL-Degrading Enzyme of *Sp. multivorum* OJ10

ε-PL-tolerant bacteria were isolated from soil samples by enrichment culture using ε-PL as a main carbon source (Kito et al. 2002a). The enrichment culture medium contained 0.1–1 mg ml^{-1} ε-PL, in the light of MICs for various bacteria (below 100 μg ml^{-1}) (Shima et al. 1984; Hiraki 2000). The strain OJ10, which exhibited the highest ε-PL-degrading activity, was selected and identified to be *Sp. multivorum*. *Sp. multivorum* OJ10 can grow in the presence of 10 mg ml^{-1} ε-PL without a prolonged lag phase, and complete growth inhibition is not observed even at 100 mg ml^{-1}. Shima et al. (1984) reported that MICs for bacteria are generally 1–8 mg ml^{-1}, and those of fungi and yeast are about 120–250 mg ml^{-1}. This clearly indicates the contribution of ε-PL-degrading activity to ε-PL-tolerance of *Sp. multivorum* OJ10.

ε-PL-degrading activity of *Sp. multivorum* OJ10 is detected in the cell-free extract prepared from the bacterial cells. The degrading activity of *Sp. multivorum* OJ10 is

also found in the cells cultivated in nutrient medium without ε-PL, suggesting that ε-PL-degrading enzyme is a constitutive enzyme. Using the buffer containing dithiothreitol and $CoCl_2$, the degrading enzyme was purified to homogeneity. The purified enzyme is a monomeric structure of 80 kDa. The ε-PL-degrading enzyme displays a high sensitivity to *p*-chloromercurienzoate, *N*-ethylmaleimide, 5,5′-dithiobis (2-nitrobenzoic acid), Ag^+, Hg^{2+}, EDTA, α,α′-dipyridyl, *o*-phenanthroline, Tiron, and *N*,*N*′-diethyldithiocarbamate; the activity is activated by Co^{2+} and Ca^{2+}.

The purified enzyme catalyzes exo-type degradation of ε-PL, with the release of L-lysine. The apparent *K*m values for ε-PL and L-lysyl-*p*-nitroanilide are 186 μM (assuming $n = 30$) and 2.08 mM, respectively. The purified ε-PL-degrading enzyme catalyzes the hydrolysis of various substrates (Table 1). However, α-PL ($n = 186$–382) is inert as the substrate. When Lys-Phe-His-Gln-Lys-His-His-Ser-His-Ser-His-Arg-Gly-Tyr is incubated with the purified enzyme, L-Lys, L-Phe, L-His, and L-Gln are released in that order. The ε-PL-degrading enzyme of *Sp. multivorum* OJ10 is similar to aminopeptidases Y and Co from *Saccharomyces cerevisiae* (Yasuhara et al. 1994; Achstetter et al. 1982), which catalyze the efficient hydrolysis of Lys-, Arg-, and Leu-4-methylcoumaryl-7-amides and

Table 1 Substrate specificity of ε-PL-degrading enzyme from *Sp. multivorum* OJ10

Substrate	Concentration (mg or mM)	Relative activity (%)
ε-PL	4 mg	2
α-PL	4	0
L-Lys-*p*-nitroanilide	5 mM	100
L-Arg-*p*-nitroanilide	5	55
L-Leu-*p*-nitroanilide	5	122
L-Ala-*p*-nitroanilide	5	53
L-Pro-*p*-nitroanilide	5	0
Lys-Lys	1	6
Lys-Lys-Lys	1	3
Lys-Lys-Lys-Lys	1	0.8
Lys-Ala	1	3
Lys-Asp	1	15
Lys-Gly	1	9
Lys-Leu	1	17
Lys-Met	1	5
Lys-OMe	1	18
Lys-amide	1	14
Ala-Lys	1	4
Gly-Lys	1	2
Met-Lys	1	7
Arg-Arg-Lys-Ala-Ser-Gly-Pro	1	1
Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser-amide	1	4
Lys-Glu-Glu-Ala-Glu	1	0.4
His-Gly-Lys	1	1
Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr	0.5	4

Lys-, Arg-, and Leu-*p*-nitroanilides, respectively, and are activated by Co^{2+}. Thus, the ε-PL-degrading enzyme of *Sp. multivorum* OJ10 probably belongs to a group of cobalt-activated aminopeptidases. ε-PL might not be a physiological substrate for *Sp. multivorum* OJ10, and the enzyme activity is not inducibly formed by ε-PL. Through the enrichment culture using ε-PL, *Sp. multivorum* OJ10 possessing an aminopeptidase catalyzing ε-PL degradation could survive and be selected as an ε-PL-tolerant strain.

3.1.2 ε-PL-Degrading Enzyme of *Chryseobacterium* sp. OJ7

Chryseobacterium sp. OJ7 was also isolated by enrichment culture using ε-PL (0.1–1 mg ml^{-1}) (Kito et al. 2003). The complete growth inhibition of the bacterium is not observed even in the presence of 100 mg ml^{-1}. *Chryseobacterium* sp. OJ7 constitutively produces and secretes ε-PL-degrading enzyme in the culture medium. The enzyme activity is not contained in the cell-free extract and cell membrane fraction prepared from the bacterial cells. From the culture filtrate, the ε-PL-degrading enzyme was purified to homogeneity and characterized. The purified enzyme is a dimmer of 19.5 kDa-subunit. The enzyme activity is inhibited by Hg^{2+}, Ag^{+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, *o*-phenanthroline, α,α′-dipyridyl, *p*-chloromercuribenzoate, EDTA, 8-hydroxyquinoline, and phenylmethylsulfonyl fluoride, suggesting the importance of Cys residue(s) and metal ion(s) for the enzyme activity.

The purified enzyme from *Chryseobacterium* sp. OJ7 predominantly catalyzes the endo-type degradation of ε-PL (Fig. 2), in contrast to those of *Sp. multivorum* OJ10 (Kito et al. 2002a). The enzyme partially catalyzes the release of the terminal lysine residue of ε-PL. The exo-type degradation is probably derived from the primary structure of ε-PL, a homo-poly amino acids. When horseradish peroxidase or casein is incubated with the purified enzyme, the fragmentation of both proteins is observed. The lysyl endopeptidase of *Achromobacter lyticus* (Masaki et al. 1981) does not hydrolyze ε-PL. The purified ε-PL-degrading enzyme catalyzes the hydrolysis of the following compounds, and the release of *p*-nitroaniline or L-lysine is observed: Lys-, Ala-, Leu-, Pro-, and Arg-*p*-nitroanilides, Lys-Lys, Lys-Lys-Lys, Lys-Lys-Lys-Lys, Met-Lys, Lys-Ala, Lys-Asp, Lys-Leu, Lye-Met, Lys-amide, Lys methylester, and Lys-4-methylcoumaryl-7-amide. α-PL with molecular mass of 1,000–4,000 and 4,000–15,000 is not hydrolyzed by the purified enzyme. Gly-His-Lys, Gly-Lys, Ala-Lys, Lys-Gly, ε-*N*-Boc-Lys are inert as substrates. Thus, the adventitious possession of proteases, exhibiting high ε-PL-degrading activity, enables *Sp. multivorum* OJ10 to grow well in the presence of ε-PL.

Commercially available proteases such as Protease A, Protease P, and Peptidase R from *Aspergillus oryzae*, *A. melleus*, and *Rhizopus oryzae*, respectively (all supplied by Amano Enzyme Co., Japan), catalyze typical endo-type degradation of ε-PL as shown in Fig. 1b (Kito et al. 2002a). The ε-PL-degrading activity of these proteases might contribute to the high MIC values of ε-PL for fungi.

3.2 ε-PL Degradation by ε-PL-Producing Microorganisms

ε-PL is industrially produced by fermentation process using *S. albulus* and used as a food additive due to its high antimicrobial activity (Shima and Sakai 1977; Shima et al. 1982, 1984). *S. albulus* produces and accumulated ε-PL efficiently in the culture medium at pH 4.2–4.5, while ε-PL is immediately degraded by this strain at pH 5.0–8.0 (Shima et al. 1983; Hiraki et al. 1998; Kahara et al. 2001). The presence of ε-PL-degrading enzyme is disadvantageous for industrial ε-PL production by *S. albulus*. Therefore, in the ε-PL fermentation using *S. albulus*, the time course of ε-PL-degrading activity was investigated (Kito et al. 2002b).

The biological activity of ε-PL is known to be dependent on its molecular size (Shima et al. 1984). L-Lysine polymers with more than nine L-lysine residues show severe inhibition of microbial growth, but the ε-octamer of L-lysine shows negligible antimicrobial activity. The precursor of ε-PL biosynthesis has been identified as L-lysine by an incorporation experiments using [^{14}C]-lysine in *S. albulus* (Shima et al. 1983). Moreover, it has been reported that ε-PL might be produced by nonribosomal peptide synthetase (NRPS) (Kawai et al. 2003; Saimura et al. 2008). NRPSs are multifunctional enzymes consisting of semiautonomous domains that synthesize numerous secondary metabolites (Schwarzer et al. 2003; Mootz et al. 2002; Marahiel et al. 1997). Amino acids are first activated by ATP to produce adenylate which, being unstable, is subsequently converted to a thioester. The thioesterified amino acid is then incorporated into the peptide as another substrate. The order and number of modules of an NRPS system determine the sequence and length of the peptide product. However, it is difficult to explain the diversity of polymerization degree (n = 25–35) of ε-PL produced by *S. albulus*, on the basis of NRPS system. These raise a question of whether ε-PL-degrading enzyme of *S. albulus*, rather than NRPS system, might be involved in generating the diversity of polymerization degree of ε-PL. Therefore, the identification and characterization of ε-PL-degrading enzyme(s) of *S. albulus* had been important subjects for a long time. Kito et al. (2002b) found ε-PL-degrading activity in the membrane fraction of *S. albulus* and characterized the purified enzyme.

3.2.1 Time Course of ε-PL-Degrading Activity During ε-PL Production by *S. albulus*

The ε-PL-degrading activity of *S. albulus*, cultivated in the medium containing glucose, polypeptone, yeast extract, and mineral salts, was measured (Kito et al. 2002b). Under this culture conditions, in a pH range of 5.0–7.0, no accumulation of ε-PL is observed in the culture medium (Fig. 3a). Instead, high level of ε-PL-degrading activity is detected in the membrane fraction of *S. albulus* cells. When *S. albulus* was cultivated in the medium containing glucose, ammonium sulfate, yeast extract, and mineral salts (Fig. 3b), the pH of culture broth was maintained in a range of 3.0–4.0 after 48 h. ε-PL-degrading activity of the membrane fraction

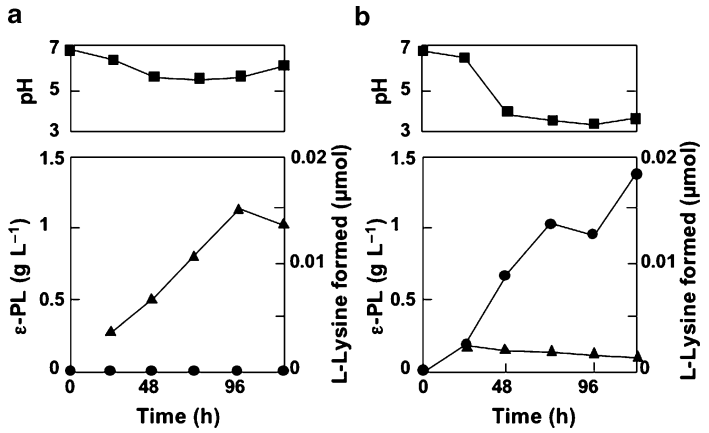

Fig. 3 ε-PL production and ε-PL-degrading activity in the course of cultivation of *S. albulus*. ε-PL-degrading activity of the membrane fraction prepared from *S. albulus* is indicated as the formed L-lysine from ε-PL. *Circles* ε-PL; *triangles* ε-PL-degrading activity of membrane fraction; *squares* pH of the medium

gradually decreases, and ε-PL accumulates in the culture medium. Thus, ε-PL production seems to be inversely related with the ε-PL-degrading activity.

3.2.2 Characterization of ε-PL-Degrading Enzyme from *S. albulus*

Most of ε-PL-degrading activity (>99%) of *S. albulus* is localized in the membrane faction (Kito et al. 2002b). Addition of 1.2 M NaSCN into the purification buffer effectively solubilizes the enzyme from the membrane fraction. Triton X-100, Tween 80, Tween 20, Brij 35, CHAPSO, and CHAPS are not effective at various concentrations on solubilization of ε-PL-degrading enzyme. The addition of 1 mM Zn^{2+} to the solubilizing buffer enhances the recovery of enzyme activity. The purified enzyme gives a single band on SDS–PAGE with a subunit molecular mass of 54 kDa. The enzyme activity is severely inhibited by *o*-phenanthroline, and can be restored in the presence of Mg^{2+}, Ca^{2+}, Fe^{3+}, and Zn^{2+}. Thus, ε-PL-degrading enzyme of *S. albulus* belongs to Zn-containing proteases.

The purified ε-PL-degrading enzyme from *S. albulus* shows the highest activity around pH 7.0, whereas the enzyme activities under acidic conditions are considerably low. In the report by Kahara et al. (2001), the optimum pH of ε-PL fermentation is around 4.0, while an increase in the pH of culture broth results in the degradation of ε-PL produced. Glucose feeding is inevitable because glucose was the energy source of ε-PL synthesis, and the pH of the culture broth is maintained at around 4.0 by adding glucose. The need to strictly control the pH of the culture broth around pH 4.0 during the fermentation process to avoid ε-PL degradation is consistent with the optimum pH profile of ε-PL-degrading enzyme.

To investigate the mode of ε-PL degradation, the purified enzyme was incubated with ε-PL, and the time course of L-lysine formation and the lengths of L-lysine polymers were analyzed (Kito et al. 2002b). In the initial stage of the incubation, various lengths of L-lysine polymer are detected, and the amount of L-lysine increases proportionally with time. In the second stage, in which almost all ε-PL is consumed, the lengths of L-lysine polymers gradually decrease, and the velocity of L-lysine formation is slower than that during the initial stage. This indicates that longer forms of L-lysine polymers serve as more suitable substrates than shorter forms. Further incubation leads to the entire degradation of ε-PL. Thus, during ε-PL fermentation process by *S. albulus*, the ε-PL-degrading enzyme is probably not involved in controlling the length of L-lysine polymers.

L-Lysyl-*p*-nitroanilide, L-arginyl-*p*-nitroanilide, and L-leucyl-*p*-nitroanilide are efficiently hydrolyzed by the degrading enzyme. Regarding oligopeptides in which the N-terminal is L-lysine residue such as Lys-Lys, Lys-Lys-Lys, Lys-Lys-Lys-Lys, Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser-amide, and Lys-Gln-Ala-Gly-Asp-Val, incubation with the enzyme results in the release of L-lysine. Gly-His-Lys, Arg-Arg-Lys-Ala-Ser-Gly-Pro, Leu-Met-Tyr-Pro-Thr-Leu-Lys, and α-poly-L-lysine (molecular weight of 1,000–4,000) are inert as substrates, and L-lysine is not released. The enzyme exhibits no lysyl endopeptidase activity for peptides in which the L-lysine residue is embedded within. Therefore, the purified ε-PL-degrading enzyme is a member of aminopeptidase requiring metal ion as in the case of ε-PL-degrading enzyme from *Sp. multivorum* OJ10 (Kito et al. 2002a). Aminopeptidases Y and Co from *S. cerevisiae* are Co^{2+}-activated enzyme that hydrolyze L-lysyl-, L-arginyl-, and L-leucyl-7-amide, and L-lysyl-, L-arginyl-, and L-leucyl-*p*-nitroanilide, respectively (Achstetter et al. 1982; Yasuhara et al. 1994). An aminopeptidase from *Streptomyces griseus*, Ca^{2+}-activated zinc aminopeptidase, which is similar to amiopeptidase Y in primary structure, efficiently catalyzes the release of L-alanine, L-valine, L-proline, and L-lysine (Ben-Meir et al. 1993; Maras et al. 1996). The lysine aminopeptidase of *Aspergillus niger* acts preferably on substrates containing an N-terminal L-lysine and is a Zn^{2+} enzyme (Basten et al. 2001). The cell wall-associated aminopeptidase of *Lactobacillus helveticus*, which is activated by Co^{2+}, Ca^{2+}, and Mn^{2+}, acts on L-lysyl-*p*-nitroanilide as the most suitable substrate (Blanc et al. 1993). However, in all the reports, ε-PL was not tested as a substrate. Therefore, we cannot discuss the ability of these aminopeptidases to degrade ε-PL.

3.2.3 Correlative Distribution of ε-PL-Degrading and ε-PL-Producing Activities

In the course of studies on ε-PL-degrading enzymes of *S. albulus*, the distribution of ε-PL-degrading enzyme in various *Streptomyces* strains was examined (Kito et al. 2002b). Among the tested *Streptomyces* strains, the membrane fraction prepared from *S. virginiae* NBRC12827 and *S. norsei* NBRC15452 exhibits high ε-PL-degrading activity, the mode of ε-PL degradation being exo-type (Fig. 4). Membrane fraction of other strains such as *S. melanogenesis* NBRC12890 and *S. purpurascens*

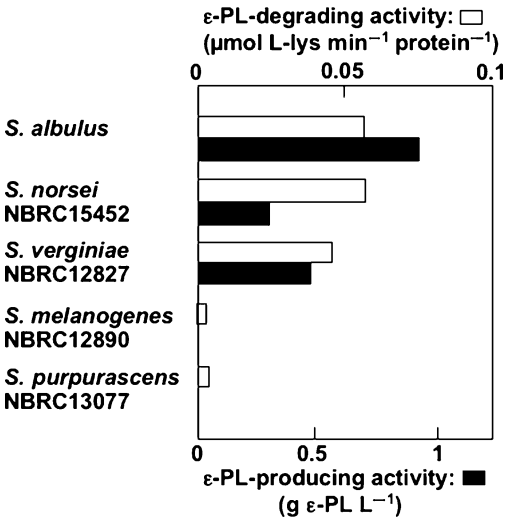

Fig. 4 Distribution of ε-PL-degrading enzyme and ε-PL-producing activity in *Streptomyces* strains. ε-PL-degrading activity of membrane fraction of cells is indicated as the formed L-lysine from ε-PL. The following strains barely exhibited ε-PL-degrading activity: *S. purpurascens* NBRC13077, *S. griseus* NBRC3237, *S. griseolus* NBRC3402, *S. phaeochromogenes* JCM4070, *S. griseoviridis* NBRC12874, *S. antibioticus* NBRC12838, and *S. melanogenes* NBRC12890

NBRC13077 exhibits considerably low ε-PL-degrading activity (<0001 μmol-L-lysine min^{-1} mg $protein^{-1}$). When ε-PL-producing activity of these *Streptomyces* strains was also investigated, an interesting correlative distribution between ε-PL-degrading activity and ε-PL-producing activity was found. Namely, *S. virginiae* NBRC12827 and *S. norsei* NBRC15452 produced ε-PL in the culture medium. The molecular mass of the ε-PL produced by both strains is almost the same as that produced by *S. albulus*. In *Streptomyces* strains showing low ε-PL-degrading activity, ε-PL-producing activity is not detected at all. This close correlation suggests a physiological role of the ε-PL-degrading enzyme. Excreted ε-PL is adsorbed to the cell surface by virtue of its ionic affinity (Shima et al. 1984), and consequently microorganisms, sensitive to ε-PL, cannot grow due to this adsorbent action. The ε-PL-degrading enzymes of ε-PL producers might play a role in self-protection against ε-PL, and this is suggested by the fact that ε-PL-degrading enzymes are found in ε-PL-tolerant microorganisms such as *Sp. multivorum* OJ10 (Kito et al. 2002a) and *Chryseobacterium* OJ7 (Kito et al. 2003). As for *S. albulus*, good growth is observed even in the presence of 100 mg ml^{-1} ε-PL. Generally, the MICs of ε-PL for bacteria and for fungi and yeasts are 1–8 and −250 μg ml^{-1}, respectively (Shima et al. 1983; Hiraki 2000). The difference in MICs might derive from the substrate specificity of proteases or cell surface conditions.

4 Molecular Genetic Analysis of ε-PL-Degrading Enzyme of *S. albulus*

The ε-PL-degrading enzyme of *S. albulus* probably plays a role in self-protection against the ε-PL produced by the microorganism itself as described above. Recently, the *pld* gene encoding ε-PL-degrading enzyme of *S. albulus* has been

cloned and analyzed (Hamano et al. 2006). Using a knockout mutant, in which the *pld* gene is inactivated, the biological function of ε-PL-degrading enzyme is investigated (Hamano et al. 2006).

4.1 *Cloning and Analysis of the* pld *Gene for* ε*-PL-Degrading Enzyme of* S. albulus

The N-terminal amino acid sequence of ε-PL-degrading enzyme purified from *S. albulus*, ADFTPGA, did not show significant similarity to the N-terminal sequences obtained in available databases. Therefore, the *pld* gene was cloned from *S. albulus* using an oligonucleotide probe designed based on the N-terminal amino acid sequence (Hamano et al. 2006). The *pld* gene encodes a 495-amino-acid protein with a calculated molecular mass of 53,931 kDa; this is in good agreement with the value obtained from the purified ε-PL-degrading enzyme (Kito et al. 2002b). In the primary structure, a Zn^{2+}-binding motif sequence was found, indicating that ε-PL is classified as a kind of Zn^{2+}-containing aminopeptidase. The putative signal peptide of secretory proteins, observed in both prokaryotic and eukaryotic cells (Nilsson and con Heijne 1991), is also found in the deduced N-terminal sequence of the ε-PL-degrading enzyme of *S. albulus*. However, the ε-PL-degrading activity is detected in the membrane fraction of *S. albulus* cells but not in the culture filtrate (Kito et al. 2002b). Probably, the degrading enzyme tightly binds to the cell membrane surface. The database search reveals that *S. avermitilis* MA-4680 and *S. coelicolor* A3(2) possess the genes *SAV1675* and *SCO6736*, respectively, and both genes are homologous to the *pld* gene and are annotated as putative metalloprotease genes.

In the immediate upstream region of the *pld* gene, the genes encoding two peptidases, X-Pro dipeptidyl-peptidase and carboxypeptidase, are located. The *pld* gene organization of the peptidase-rich region of *S. albulus* genome is identical to that of the *S. avermitilis* MA-4680 genome, and it differs from the gene organization observed in *S. coelicolor* A3(2). Using *S. avermitilis* NBRC14893, MIC of ε-PL for the strain was examined. *S. avermitilis* is more sensitive to ε-PL (MIC of 6.25 μg ml^{-1}) than *S. albulus*, and ε-PL-producing activity is not observed using the strain. Additional analysis of flanking regions surrounding the peptidase-rich region is also performed. The gene organization adjacent to the peptidase-rich region of *S. albulus* genome is different from those of *S. avermitilis* MA-4680 and *S. coelicolor* A3(2) genomes, suggesting that the peptidase gene-rich region of *S. albulus* has been evolutionally developed for self-protection to ε-PL.

4.2 *Biological Function of the* pld *Gene in* S. albulus

By sequencing analysis of the *pld* gene of *S. albulus*, the homologous genes, *SAV1675* and *SCO6736*, are found in *S. avermitilis* MA-4680 and *S. coelicolor*

A3(2), respectively. To confirm whether the *pld* gene plays a role for ε-PL-tolerance of *S. albulus*, a mutant (*S. albulus* CRM001) in which the *pld* gene is inactivated, was constructed (Hamano et al. 2006), and its tolerance against ε-PL and ε-PL-degrading activity was examined.

In the MIC study, the MIC value of ε-PL for *S. albulus* CRM001 is significantly lowered to 12.5 μg ml^{-1} in comparison with that of the parent strain, 50 μg ml^{-1}. This clearly indicates the involvement of the *pld* gene in ε-PL-tolerance of the parent *S. albulus* strain. However, by prolonging cultivation time, the mutant CRM001 showed tolerance against high concentration of ε-PL. Therefore, in addition to the *pld* gene, other gene(s) probably confer tolerance against ε-PL. After incubation of ε-PL with *S. albulus* CRM001 cells, ε-PL is completely degraded on HPLC analysis, also demonstrating that the mutant CRM001 produces other enzyme(s) degrading ε-PL. The amount of L-lysine released from ε-PL by *S. albulus* CRM001 cells is reduced in comparison with the parent *S. albulus*; the aminopeptidase activities of *S. albulus* CRM001 and the parent strain are 2.3 and 5.9 units mg^{-1}, respectively. These results are in good agreement with the fact that the *pld* gene encoding the aminopeptidase for ε-PL is inactivated in the strain CRM1. The detailed HPLC analysis of ε-PL degradation using the parent *S. albulus* cells reveals that, in addition to the exo-type aminopeptidase, *S. albulus* possesses ε-PL-degrading enzyme(s) catalyzing end-type degradation of ε-PL. For a sufficient ε-PL degradation leading to ε-PL-tolerance phenotype, probably, *S. albulus* requires not only the *pld* gene but also other unknown gene(s).

5 Conclusion

ε-PL is applied as a food additive due to its high antimicrobial activity, and its further applications as a biodegradable cationic polymer have been developed. ε-PL is chemically stable; no degradation is observed after boiling at 100°C for 30 min or autoclaving at 120°C for 20 min (Hiraki 2000). The safety of ε-PL has been confirmed as a food preservative. However, there had been no reports on biodegradation of ε-PL, until the discovery of ε-PL-degrading enzymes in ε-PL-tolerant bacteria by Kito et al. (2002a). The ε-PL-degrading enzymes of ε-PL-tolerant bacteria are characterized as exo- and endo-type peptidase, catalyzing the hydrolysis of various peptides and artificial substrates. The degrading activity of the peptidases from ε-PL-tolerant bacteria is not specific for ε-PL. The adventitious possession of peptidases catalyzing ε-PL degradation enables ε-PL-tolerant bacteria to grow in the presence of high concentration of ε-PL.

ε-PL-degrading activity is also detected in the membrane fraction of ε-PL-producing *S. albulus*. *Streptomyces* strains exhibiting high ε-PL-degrading activity can produce ε-PL in their culture medium. This correlative distribution of ε-PL-degrading activity and ε-PL-producing activity suggests the contribution of ε-PL-degrading activity to self-protection against ε-PL. The purified ε-PL-degrading enzyme from *S. albulus*, a representative ε-PL-producing strain, is characterized as zinc-containing

aminopeptidase. Molecular genetic analysis of the *pld* gene encoding ε-PL-degrading enzyme revealed that, at least, two enzymes are involved in the ε-PL degradation for self-protection against ε-PL in *S. albulus*. Recently, Yamanaka et al. (2008) have found ε-PL synthetase (Pls) activity in the membrane fraction of *S. albulus* and verified as a novel NRPS on the basis of the structural analysis. In the flanking region of the *pls* gene, the gene encoding a putative aminopeptidase, probably catalyzing ε-PL degradation, has been found.

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Biotechnological Production of Poly-Epsilon-L-Lysine for Food and Medical Applications

Kazuya Yamanaka and Yoshimitsu Hamano

Contents

Abstract Poly-ε-L-lysine (ε-poly-L-lysine, ε-PL) consisting of 25–35 L-lysine residues in isopeptide linkages is one of only two amino-acid homopolymers known in nature. ε-PL was found as a Dragendorff-positive substance produced by *Streptomyces albulus* isolated from soil more than 30 years ago. Because of its strong

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antimicrobial activity and safety, ε-PL has been industrially produced and widely used as a natural food preservative. To comply with growing demand for ε-PL in food applications, numerous studies aimed at improving the productivity of ε-PL have been carried out. In addition, there has been great interest in medical and other applications of ε-PL and its derivatives. In this review, the microbial production of ε-PL, its antimicrobial activity and safety, and various applications are comprehensively described.

1 Introduction

Two amino-acid homopolymers comprising a single amino acid building block are known in nature: γ-poly-glutamic acid (γ-PGA) and ε-poly-L-lysine (ε-PL). The latter, ε-PL, consists of 25–35 L-lysine residues with isopeptide linkage between its ε-amino and α-carboxyl group. This characteristic polymer was discovered as a Dragendorff-positive substance produced by actinomycete *Streptomyces albulus* strain 346 isolated from soil (Shima and Sakai 1977, 1981a, b). ε-PL shows strong antimicrobial activity against a wide spectrum of microorganisms including bacteria, fungi, and also some kinds of viruses (Shima et al. 1984). Since the safety of ε-PL was demonstrated in experiments using rats (Hiraki 1995, 2000), it has been widely used as a natural food preservative in a number of countries including Japan, the United States, and Korea.

To comply with growing demand for ε-PL in food applications, since the discovery of ε-PL in the culture filtrate of *S. albulus*, a large number of studies aimed at improving the productivity of ε-PL have been reported. For example, Hiraki et al. (1998) derived a ε-PL high-producer by nitrosoguanidine treatment. The productivity of the resultant mutant was ten times higher than that of the wild strain. At present, ε-PL is industrially produced by using a second-generation mutant of *S. albulus*.

Diverse amino-acid polymers have been chemically synthesized to find attractive bioactive substances. Among them, it is well known that α-poly-L-lysine (α-PL) shows various applications in the life science field. However, because α-PL shows some toxicity (Sela and Katchalski 1959), naturally occurring ε-PL is expected to be an ideal alternative substance to synthetic α-PL in medical use. In fact, due to its safety and edible features, ε-PL and its derivatives have attracted great interest for use in medical and other applications. Gaining further understanding of the unique physicochemical properties of ε-PL, including its water soluble, polycationic, nontoxic, and edible features, is necessary to develop more biomedical and industrial applications. Similarly, further improvement of the microbial production system is needed. In this review, the microbial production of ε-PL, its antimicrobial activity and mechanism, safety, and applications in food are comprehensively described. Additionally, some of the notable research and practical uses in medical and other applications are reviewed.

2 Microbial Production of ε-PL

As mentioned above, ε-PL was discovered as an extracellular material produced by *S. albulus* No. 346, now designated *S. albulus* NBRC 14147, isolated from soil more than 30 years ago (Shima and Sakai 1977). To date, a number of ε-PL producers including the *S. albulus* strains have been isolated from soil. Surprisingly, the distribution of ε-PL producers was quite limited among bacteria in the family *Streptomycetaceae* and ergot fungi (Nishikawa and Ogawa 2002). The productivities of ε-PL from most of these strains are much lower than that of *S. albulus* 346. Therefore, most studies on the microbial production of ε-PL have been focused on *S. albulus* 346 or its derivatives.

2.1 *ε-PL Production in* S. albulus *No. 346 (Wild-Type)*

In the first study with the wild-type strain, the shake flask cultures in basal medium containing 5% glycerol, 1% $(NH_4)_2SO_4$, 0.5% Yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.003% $FeSO_4 \cdot 7H_2O$, 0.004% $ZnSO_4 \cdot 7H_2O$, and 1/50M KH_2PO_4–Na_2HPO_4 buffer (pH 6.8) typically yielded 0.2 g/l of ε-PL after cultivation for 96 h at 30°C. In this fermentation process, the accumulation of ε-PL was observed only after cell growth reached the stationary phase, and a low-pH environment is needed for ε-PL production. The substitution of a nitrogen source to organic nitrogen compounds such as polypeptone and casamino acid did not induce the low-pH environment, and consequently inhibited ε-PL production, showing that the restricted nitrogen source was required for ε-PL production. Although nitrate compounds inhibited ε-PL production, sulfates were required. Based on these results, Shima and coworkers developed a ε-PL production system with the mycelium in the buffer containing glucose and $(NH_4)_2SO_4$ (Shima et al. 1983). By controlling the acidic pH condition (pH 4.0–4.5), high productivity of ε-PL (4–5 g/l) was observed in this system.

2.2 *Improvement of ε-PL Production Using a Mutant Strain of* S. albulus

Shima et al. have reported that the L-lysine molecule is directly utilized in ε-PL biosynthesis (Shima et al. 1983). In most bacteria, L-lysine is biosynthesized by the amino-acid biosynthetic pathway from L-aspartic acid (aspartate pathway, see chapter "Biochemistry and Enzymology of Poly-Epsilon-L-Lysine Biosynthesis" by Hamano). The first two enzymes in this pathway are aspartokinase (Ask) (EC.2.7.2.4), which catalyzes the phosphorylation of L-aspartic acid to produce L-4-phospho aspartic acid, and aspartate semialdehyde dehydrogenase (Asd) (EC.1.2.1.11), which reduces L-4-phospho aspartic acid into L-aspartate 4-semialdehyde. These two key enzymes are subjected to complex regulation by the end-product amino acids. Mutants in

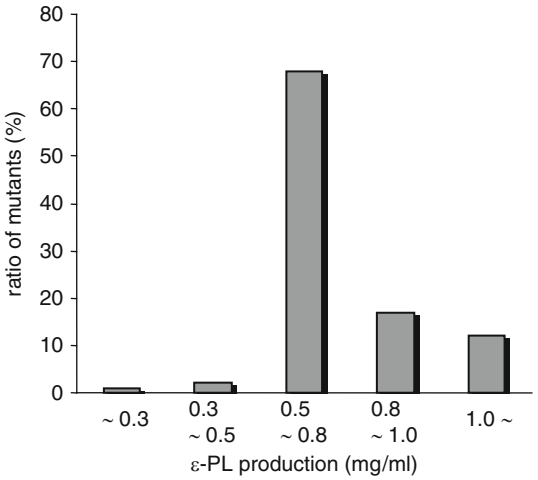

Fig. 1 Distribution of ε-PL productivities of AEC plus glycine-resistant mutants derived by NTG treatment (Hiraki et al. 1998). One-hundred and eighty-eight mutants were subjected to test tube cultivation in medium A at 30°C. After 4 days, the ε-PL productivities were measured

Corynebacterium glutamicum and *Brevibacterium flavum* resistant to the L-lysine analogue are known to produce a large amount of L-lysine due to resistance to the feedback-inhibition of the two enzymes. Similarly, a mutant strain of *S. albulus* 346 resistant to the L-lysine analogue was also expected to be a high-producer of ε-PL, because L-lysine, which is the biosynthetic precursor of ε-PL, must be accumulated.

Although Hiraki and coworkers attempted to construct such a mutant, the wild-type was, in itself, resistant to the L-lysine analogue such as an *S*-(2-aminoethyl)-L-cysteine (AEC) (Hiraki et al. 1998). However, they revealed that the growth of the wild-type was fully inhibited by the addition of glycine. Therefore, they screened mutants resistant to AEC plus glycine (Hiraki et al. 1998). As shown in Fig. 1, 99% of the AEC plus glycine-resistant mutants showed high productivities, and one of them, 11011A, showed the highest productivity of 2.11 mg/ml in a test tube culture, which was tenfold higher than that of the wild-type strain. Moreover, the Ask activity of the mutant strain 11011A was about twofold higher than that of the wild-type, and was not inhibited by the addition of L-lysine. Finally, in a 3-l jar fermentor with feeding glucose and the pH of the medium maintained at pH 4.5–4.2, 11011A produced 20 g/l of ε-PL with an 8.9% yield against the consumed glucose after 120-h cultivation.

2.3 Optimization of ε-PL Production for Commercial Supply

To optimize production for commercial supply, further investigation of the microbial production of ε-PL has been carried out. Using *S. albulus* 410 (S410), which is one of the ε-PL high producers, Kahar et al. (2001) have demonstrated that a strict pH condition and glucose concentration was important for ε-PL fermentation. As mentioned earlier, ε-PL is known to accumulate under an acidic pH condition (pH 4.0–4.5). Therefore, the enhancement of ε-PL production was evaluated by means

of a strict pH control strategy. The optimized fermentation condition was divided into two phases. In the first phase, the cell growth was accelerated by maintaining the pH higher than 5.0. In the second phase, the pH of the culture medium was maintained around 4.0, at which the highest ε-PL productivity was observed. In addition, because the yield of ε-PL against consumed glucose and its productivity were affected by the residual glucose concentration of the culture medium, the glucose concentration was strictly kept at about 10 g/l. In their strategy, ε-PL productivity was enhanced to 48.3 g/l. Thus, since the first study, the ε-PL productivity of *S. albulus* has been significantly improved by using these biotechnological techniques (Table 1). At present, ε-PL has been industrially produced by a fermentation process using a mutant strain derived from 11011A and the pH control strategy (Chisso Corp.).

3 Biological Activities of ε-PL

ε-PL shows strong antimicrobial activity against both Gram-positive and Gram-negative bacteria, fungi, and some kinds of viruses. The most plausible antimicrobial mechanism of ε-PL is physical disruption of the microbial cytoplasmic membrane following electrostatic absorption. Microbicidal action then occurs, and its activity is comparable to that of disinfectants widely used in medical and industrial fields.

3.1 Antimicrobial Activities and Antiphage Activity of ε-PL

ε-PL shows strong antimicrobial activity against a wide spectrum of microorganisms (Shima et al. 1984). The minimum inhibitory concentrations (MICs) for most bacteria were 1–8 μg/ml, although those for yeast and fungi tended to be slightly higher. For example, MIC for *Staphylococcus aureus* and *Candida albicans* was 4 and 128 μg/ml, respectively (Shima et al. 1984). ε-PL also shows high thermostability; the antimicrobial activity of ε-PL was not affected at all after the heat treatment at 120°C for 20 min (Hiraki 1999). Although ε-PL showed strong and broad antimicrobial activity, it did not directly show inhibitory activity for bacterial spore germination (Hiraki 1999). In the presence of 500 μg/ml ε-PL, inactivation of bacteriophages belonging to the long-tail and noncontractile morphological type was observed (Shima et al. 1982). Although a large number of studies on the antimicrobial activities of ε-PL have been reported, there have been an insufficient number of studies on its antiphage activities.

3.2 Antimicrobial Mechanism of ε-PL

Shima et al. (1984) also investigated the relationship between the chain length of ε-PL and its antimicrobial activity for *Escherichia coli* K-12. ε-PLs with more

Table 1 Improvement of microbial production of ε-PL

Year	*S. albulus* strains	Culture method (initial nutrients)	Maximum productivity (culture time)	Improvement (fold)	References
1977	346 (wild)	Shake flask culture in basal medium (50 g/l glycerol, 10 g/l $(NH_4)_2SO_4$, 5 g/l yeast extract)	0.2 g/l (4 days)	–	Shima and Sakai (1977)
1983	346 (wild)	Reaction with cells in citrate buffer (50 g/l glycerol, 10 g/l $(NH_4)_2SO_4$, 20 g/l citrate (pH 4–4.5))	5.0 g/l (8 days)	25	Shima et al. (1983)
1998	11011A (mutant)	Test tube culture in medium A (50 g/l glucose, 10 g/l $(NH_4)_2SO_4$, 5 g/l yeast extract)	2.1 g/l (4 days)	10.5	Hiraki et al. (1998)
		Jar fermentation in medium A with feeding glucose and pH maintenance (pH 4.2)	20 g/l (5 days)	100	
2001	S410 (mutant)	Jar fermentation in M3G medium with glucose concentration and pH controlled (10 g/l, pH 5.0→4.0) (50 g/l glucose, 10 g/l $(NH_4)_2SO_4$, 5 g/l yeast extract)	48.3 g/l (8 days)	241	Kahar et al. (2001)

than nine L-lysine residues strongly inhibited their growth, although the activity of ε-PLs with less than eight residues were reduced (>100 μg/ml). The importance of free α-amino groups of ε-PL for antimicrobial activity was demonstrated by a reduction of the activity with chemical modification of their α-amino groups. Based on a study using electron microscopy, the proposed mechanism of antimicrobial action of ε-PL was concluded to be electrostatic interaction with the microbial cell surface, followed by disorganization of the membrane and abnormal distribution of the cytoplasm, ultimately leading to physiological damage of the microbial cells (Shima et al. 1984). Muto (2003) reported that both Gram-positive and Gram-negative bacteria exposed to ε-PL were disinfected. After the exposure of bacterial cells to a low concentration of ε-PL (10 μg/ml) in 10 mM potassium phosphate buffer (pH 7.0) for 15–60 min, the reduction of survival cells (>4–5 Log) was observed in all bacterial strains tested, including *E. coli*, *B. subtilis*, and *S. aureus*. In addition, ε-PL induced leakage of cytoplasmic components from *E. coli* cells. The leakage of NADH-dependent cytoplasmic malate dehydrogenase (MDH), an enzyme of the tricarboxylic acid cycle, from ε-PL treated cells was observed, and the increase of leaked MDH activity showed good correlation with a decrease in cell survival (Muto 2009a, Fig. 2). As reported previously, polycationic compounds such as protamine interact with the cell surface membrane and induce leakage of cytoplasmic components such as ATP and β-galactosidase (Johansen et al. 1997). In the same way, ε-PL should first absorb to the cell surface and then disrupt the cytoplasmic membrane of microorganisms. After fatal damage to the membrane, cytoplasmic components are thought to leak out. Based on all of these results, it can be concluded that the antimicrobial mechanism of ε-PL is physical disruption of the cytoplasmic membrane following electrostatic absorption.

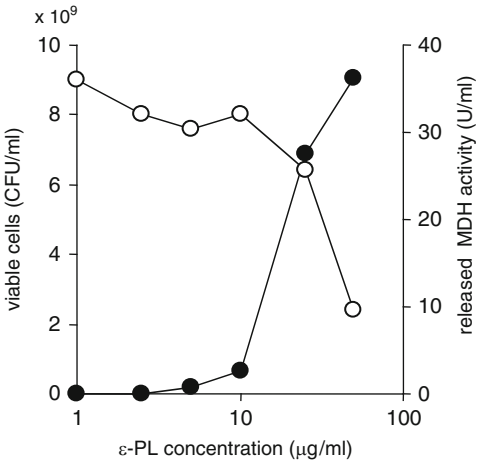

Fig. 2 Leakage of the cytoplasmic malate dehydrogenase (MDH) from *E. coli* cells exposed to ε-PL (Muto 2009a). *E. coli* cells (>9 Log CFU/ml) were exposed to ε-PL in 10 mM Tris–HCl (pH 7.0). After exposure for 1 h, the number of survival cells (*open circle*) and MDH activity leaked to supernatant (*closed circle*) were determined

3.3 Microbicidal Activities of ε-PL

Although many studies on the growth inhibitory activities of ε-PL have been reported, its microbicidal activities are poorly understood. Based on the understanding of its antimicrobial mechanism, the microbicidal activities of ε-PL have been investigated (Muto 2009a). The minimum bactericidal concentrations (MBCs) of ε-PL, which was defined as the lowest concentration of ε-PL that disinfected over 99.99% of the inoculated cells within 2 h, were determined (Table 2). The MBCs of ε-PL against all of the tested microorganisms including Gram-positive, Gram-negative bacteria, and also yeasts were <50 μg/ml and were comparable to those of disinfectants such as cetylpyridinium chloride (CPC) and 1,2-benzisothiazolin-3-one (BIT), which are widely used in medical and industrial fields. Due to its safety and edible feature, the availability of ε-PL as a bacteriocide that can be used in multiple fields including the medical one has been suggested (Muto 2009a). In addition, a clear correlation between microbial susceptibility to ε-PL and relative cell size was observed (Muto 2009a, Fig. 3). Yeast strains were also included in this consistent correlation. Thus, the microbicidal activities of ε-PL depended not on the microbial species, but directly on the microbial cell size. This phenomenon also strongly supports the conclusion proposed in the section above that the antimicrobial activity of ε-PL is caused by a physical action on the cytoplasmic membrane.

Table 2 Comparison of minimum bactericidal concentrations (MBCs) of ε-PL and different disinfectants for the selected microorganisms (Muto 2009a). MBCs were defined as the lowest concentration that disinfected over 99.99% of the inoculated cells within 2 h and were determined with 10 mM potassium phosphate buffer (pH 7.0)

Microorganisms	MBC (μg/mL)				
	ε-PL	PHMB	BAC	CPC	BIT
Gram-negative bacteria					
Escherichia coli IFO 3972	6.3	<3.1	6.3	12.5	100
Pseudomonas aeruginosa IFO 13275	<3.1	<3.1	6.3	12.5	200
Salmonella choleraesuis IFO 3163	<3.1	n.d.	n.d.	n.d.	n.d.
Klebsiella pneumoniae IFO 13277	6.3	n.d.	n.d.	n.d.	n.d.
Gram-positive bacteria					
Bacillus subtilis IFO 13719	13	n.d.	n.d.	n.d.	n.d.
Staphylococcus aureus IFO 12732	6.3	6.3	<3.1	<3.1	100
S. aureus IFO 13276	6.3	<3.1	<3.1	6.3	100
Listeria monocytogenes JCM 2873	6.3	6.3	6.3	<3.1	200
Yeast					
Saccharomyces cerevisiae IFO 10517	25	n.d.	n.d.	n.d.	n.d.
Pichia anomala IFO 0141	25	n.d.	n.d.	n.d.	n.d.
Candida albicans IFO 1594	13	6.3	12.5	12.5	200

PHMB polyhexamethylene biguanide, *BAC* benzalkonium chloride, *CPC* cetylpyridinium chloride, *BIT* 1,2-benzisothiazolin-3-one, *n.d.* not determined

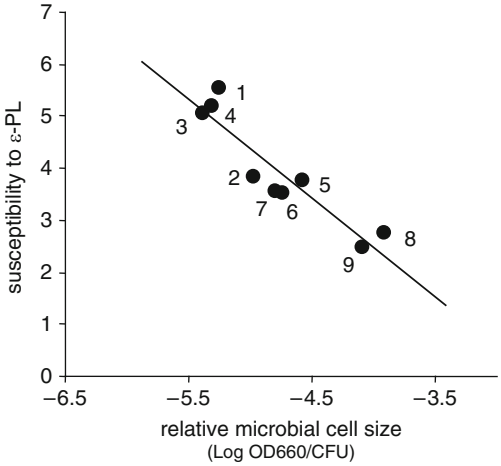

Fig. 3 Relationship between microbial susceptibility to ε-PL and relative cell size (Muto 2009a). The susceptibility to ε-PL was defined as the logarithms of the number of disinfected microbial cells by the treatment with 10 μg/ml ε-PL in 10 mM potassium phosphate buffer (pH 7.0) within 2 h. Relative microbial cell sizes were estimated from the relationship between the turbidity measured at 660 nm and the number of viable cells in microbial suspensions. 1, *Escherichia coli*; 2, *Salmonella choleraesuis*; 3, *Pseudomonas aeruginosa*; 4, *Klebsiella pneumoniae*; 5, *Bacillus subtilis*; 6, *Bacillus cereus*; 7, *Listeria monocytogenes*; 8, *Saccharomyces cerevisiae*; 9, *Pichia anomala*

3.4 Antimicrobial Profiles of ε-PL

The antimicrobial activities of ε-PL were observed in weak acidic or physiological pH conditions. ε-PL is a cationic polymer and its isoelectric point is around 9.0. The antimicrobial and antiphage activities of ε-PL are significantly weakened under the alkaline conditions, because these activities are simply based on the electrostatic interaction. Hiraki (2000) reported that the MICs for *E. coli* observed in physiological pH conditions are 25–50 μg/ml, whereas those at pH 8.0 are over 200 μg/ml. In addition, the activity was weakened with increasing ionic strength and/or the presence of anionic compounds such as metaphosphoric acid, due to loss of its cationic charge. Similarly, under the extremely low pH condition, the activities would be inhibited due to loss of the counter anionic charge for ε-PL on the microbial cytoplasmic membrane.

3.5 Safety of ε-PL

The safety of ε-PL was confirmed by experiments using rats. In a two-generation reproduction study, the nontoxic dosage level of ε-PL was concluded to be 10,000 ppm, and ε-PL at 30,000 ppm did not cause any toxicities for reproduction, neurological function, embryonic, and fetal development and growth (Neda et al.

1999). The pharmacokinetics of ε-PL in vivo were also investigated (Hiraki et al. 2003). Absorption, distribution, metabolism and excretion (ADME) studies using ^{14}C-radiorabeled ε-PL revealed that ε-PL was poorly absorbed in the gastrointestinal tract and most of the dosed radioactivity was eliminated by excretion within 168 h. Furthermore, no accumulation of ε-PL in any tissue or organ was observed by whole body autoradiography. Based on these results showing its safety, ε-PL was approved by the Japanese Ministry of Health, Labour and Welfare as a preservative in food in the late 1980s. Thereafter, ε-PL was listed in the Korea Food Additives Code and has been used in Korea. Recently, the US Food and Drug Administration indicated that they had no questions about the manufacturer's conclusions that ε-PL is generally recognized as safe (GRAS) for use as an antimicrobial agent in cooked or sushi rice at levels up to 50 mg/kg of rice (USFDA 2004).

4 Application of ε-PL

Since the safety of ε-PL has been clarified, ε-PL has been used for various applications, particularly as a food preservative. Recently, some interesting applications in medical field have been reported.

4.1 Food Preservative

ε-PL has a long history of safe use in Japan as a natural food preservative, and many food products containing ε-PL can be found in food shops and supermarkets. ε-PL has been used in various food products in Japan, including traditional Japanese dishes, boiled rice, noodles, potato salad, and cooked vegetables (Hiraki 2000). In addition, ε-PL is also used in desserts, including steamed cake and custard cream (Hiraki et al. 2003). The ε-PL formulation recipes for some commonly consumed foods are shown in Table 3 (Muto 2009b). These foods

Table 3 Recipes of the ε-PL formulation for several food products. The table was modified from that reported by Muto (2009b). ε-PL was used as a formulation including 30% sodium acetate and 20% of the other organic acids. Lifetime, period that bacterial growth was inhibited (<5 Log CFU/g)

Food products	Recipe (as a formulation)	Preservation	
		Temperature	Lifetime
Hamburger steak	Add 0.5% and mix with putty before frying	25°C	48 h
Hamburger patty	Add 2–3% and mix with putty	4°C	20 days
Boiled vegetables	Cook in the 0.8% solution	25°C	72 h
Croquette	Add 0.6% and mix before frying	25°C	72 h
Cooked fish	Cook in the 0.6% solution	25°C	48 h
Potato salad	Add 0.6% and mix	10°C	72 h
Mashed potato	Add 0.6% and mix after cooked	4°C	12 weeks

typically contain 10–500 ppm of ε-PL. For agglomerative foods such as sliced fish and fish sushi, spraying and dipping treatment with 1,000–5,000 ppm of ε-PL were effective (Hiraki 2000).

4.1.1 Improvement of ε-PL Preservative Activity by Combination with Other Additives

In general use, because the concentration of ε-PL required for food preservation is quite low due to its strong antimicrobial activity, the taste of the foods is not affected. Although ε-PL can be used alone for food preservation, the preservative activity of ε-PL is greatly improved by combining it with other food additives (Hiraki 2000). The combination of ε-PL and other food additives such as glycine, ethanol, organic acids including vinegar and lactic acid, and emulsifying agent is effective for multiple food preservation due to the synergistic effect. For preservation of raw chicken containing a high level of microbial contaminants (4–5 Log CFU/g as aerobic bacteria), dipping treatment using 450 ppm ε-PL solution containing 0.9% organic acid and 1.4% sodium acetate gave a 12-day preservative effect at 4°C, which was three times longer than that without treatment (Muto 2009a). The synergistic effect helps to reduce the total amount of preservatives and serves the additional positive effect of enhancing the preservative activity without affecting the original food taste. In fact, ε-PL is used together with these additives in most cases.

4.1.2 Efficacy for Controlling Food-Borne Pathogens

Microbially contaminated foods are estimated to cause approximately 76 million cases of illness every year in the United States (Mead et al. 1999). The availability of ε-PL as an antimicrobial agent against food-borne pathogens of *E. coli* O157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes* has been reported (Geornaras and Sofos 2005). When these three pathogens were incubated with ε-PL in tryptic soy broth supplemented with 0.6% yeast extract, 200–400 μg/ml of ε-PL inhibited their growth. Furthermore, for Gram-negative pathogens of *E. coli* O157:H7 and *S. typhimurium*, the antimicrobial activity of ε-PL was enhanced by combination with 0.25% sodium diacetate or 0.1% acetic acid. For Gram-positive pathogens of *L. monocytogenes*, *Bacillus cereus* cells and its spores, it has been reported that a combination with ε-PL and antimicrobial peptide nisin A, which is produced by *Lactococcus lactis* ssp. *lactis* (Hurst 1967) and has been used as a food preservative in the United States, shows synergistic antimicrobial activity (Badaoui Najjar et al. 2007). The combination of ε-PL with the above mentioned additives can be considered an effective all-natural formulation for controlling a wide spectrum of food-borne pathogens, spores, and spoilage organisms, although more research is needed to determine the most effective combination or conditions for each food application.

4.2 Medical and Other Applications

Diverse amino-acid polymers have been chemically synthesized to find attractive bioactive substances. Among them, α-PL has shown various applications in the life science field due to its polycationic property. In the medical field, α-PL has been used to enhance the efficacy of some interferon inducers (Levy et al. 1975; Champney et al. 1976). It has also been reported that α-PL enhances the drug permeability of Chinese hamster ovary (CHO) cells (Ryser and Shen 1980), and the polyionic complex of DNA with polycations such as α-PL is effective as a nonviral gene delivery carrier (Chiou et al. 1994; Dorudi et al. 1993). However, for practical use of these medical applications, the toxicity, biocompatibility, and biodegradability of α-PL need to be considered. α-PL has been used in medical applications mainly for its polycationic property. Because α-PL shows some toxicities (Sela and Katchalski 1959), naturally occurring ε-PL would be an ideal alternative substance to synthetic α-PL for medical use. Recently, in fact, a gene delivery system, which exhibits low cytotoxicity, has been developed using polyionic complex of DNA with a ε-PL derivative (Chisso Corp. and Nagasaki 2006).

4.2.1 Suppression of Dietary Fat Absorption

A novel application of ε-PL in medical use has been reported. Obesity is a serious disease that can lead to numerous health problems including diabetes (Hill et al. 2000). Because it is known that pancreatic lipase plays a crucial role in lipid absorption from the intestine (Duan 2000), natural products that can inhibit pancreatic lipase activity could suppress dietary fat absorption. It has been reported that highly basic proteins such as protamine, purothionin, and histone inhibit lipase activity in vitro (Tsujita et al. 1996). Kido et al. (2003) have reported that 10–100 mg/l ε-PL inhibited pancreatic lipase activity in substrate emulsions containing bile salts and phosphatidylcholine as an emulsifying agent. In addition, ε-PL was found to retain its inhibitory activity after incubation with digestive enzymes such as trypsin, α-chymotrypsin, and pepsin, due to its unusual isopeptide bonds, whereas α-PL, showing the same level of inhibitory activity, did not. Therefore, it was suggested that ε-PL would inhibit lipase in the digestive tract, in which proteins and α-PL that have the usual α-peptide bonds may be digested. Based on these results, they concluded that ε-PL would be able to suppress dietary fat absorption from the small intestine by inhibiting pancreatic lipase activity.

4.2.2 Endotoxin Remover and Other Practical Uses

Lipopolysaccharide (LPS), which is a constituent of the outer membrane of Gram-negative bacteria, is a potential contaminant of physiological fluids. Because LPS causes pyrogenic and shock reactions, it must be removed from medicines that are injected. It has been reported that endotoxins can be selectively removed by using

particles prepared by cross-linking between ε-PL and chloromethyloxirane (Hirayama et al. 1999). By applying this principle, a chromatographic resin, ε-PL-immobilized cellulose beads (ET clean, Chisso Corp.), was developed and has been widely used as an endotoxin remover in some countries, including Japan, Korea, China, India, and the EU.

A cell-culture device with ε-PL as a coating material for the surface of devices has also been developed, and showed low cytotoxicity and improvement in cell adhesion (Chisso Corp. and Nagasaki 2007). In some biomedical fields, ε-PL is also used to prevent periodontal disease. The combination with 0.02–2.0% (w/w) ε-PL and 0.1–10% (w/w) bentonite, which is a kind of clay mineral, inhibited the production of oral bacterial toxin (Lion Corp. 2006). In addition, ε-PL has been used as a component of the drug solution in disposable wipes, which are used for sanitation (Asahikasei Corp. 1999). Many other ε-PL applications have been patented. In the future, further applications of ε-PL will be developed.

5 Concluding Remarks and Future Perspectives

Since the discovery of ε-PL, numerous studies on its microbial production, antimicrobial activity, and application have been reported. ε-PL is now industrially produced and used in food and medical applications due to its polycationic property and safety. Many applications of ε-PL have been patented, and further studies on novel applications are in progress. The water-solubility, polycationic, nontoxic, edible feature, and other physicochemical properties of ε-PL are applicable to other fields, such as agriculture and environmental science. Recently, a ε-PL synthetase and its gene were identified (Yamanaka et al. 2008). The identification of this ε-PL synthetase and its gene will help to create new classes of biopolymers which will be useful in various applications as well as help to improve microbial production systems.

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Occurrence and Biosynthetic Mechanism of Poly-Gamma-Glutamic Acid

Makoto Ashiuchi

Contents

Abstract Poly-γ-glutamic acid (PGA) is an anionic polyamide biomaterial in which usually over 10,000 molecules of glutamic acid are polymerized via γ-amide linkages. PGA is found in various organisms, and essentially synthesized in a ribosome-independent manner. To date, two distinct modes for nonribosomal amino acid polymerization have been proposed: the thiotemplate mechanism and the amide ligation process. The former mode of polymerization often brings about a disagreement of the stereochemistry between a polymer produced and the substrate(s), differently from the latter mode. DL-PGA synthesis in *Bacillus subtilis* proceeds via

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the amide ligation mechanism and thus both isomers of glutamic acid can serve as substrates. The operon architecture responsible for DL-PGA synthesis occurs in the chromosome of *B. subtilis*, and all the components of the PGA synthetase complex, that is, PgsB, -C, -A, and -E, are membrane-associated. Here, we present the structural features and predicted functions of each Pgs component.

1 Introduction

Poly-γ-glutamic acid (PGA) is an anionic polyamide biomaterial, where more than 10,000 molecules of glutamic acid are polymerized via γ-amide linkages (Ashiuchi and Misono 2002b), possessing a chiral center in every glutamyl unit. Following the discovery of PGA (Kramar 1921; Ivánovics and Erdös 1937), it has been the subject of much interest in modern biopolymer study. Simple methods for detection, purification, and determination of PGA were established (Ashiuchi and Misono 2002b; Do et al. 2001), the majority of which are useful in genetic and biochemical studies (Ashiuchi and Misono 2002a). Stereochemically, three different types of PGA have been identified: the homopolymer of D-glutamic acid (D-PGA), the homopolymer of L-glutamic acid (L-PGA), and the random copolymer constituted of D- and L-glutamic acid (DL-PGA).

Regardless of its stereochemistry, PGA is nontoxic to humans and the environment, has been found to be edible (Ashiuchi and Misono 2002b), and possesses enormous potential as a new macromolecular material. As shown in Table 1, various applications of PGA have been established. As an example, cross-linked PGA exhibits an extremely high degree of water absorbency (Choi and Kunioka 1995) possibly making it a suitable substitute for nondegradable hydrogels. This would be an enormous benefit in agricultural, environmental, and biomedical applications, including biodegradable diapers, water reservoirs, and slow-release systems for drugs or fertilizers. Much is known about PGA-based flocculants (Yokoi et al. 1996; Shih et al. 2001; Taniguchi et al. 2005), with some available for wastewater treatment, water purification, and downstream processing for food and fermentation manufacturers. The industrial utilization of PGA is rapidly increasing with applications, such as a metals/radionuclides-binding agent (Ashiuchi and Misono 2002b), a protease inhibitor, a cryoprotectant (Birrer et al. 1994; Mitsuiki et al. 1998; Yokoigawa et al. 2006), a bitterness-relieving agent (Shih and Van 2001), a thickener, an animal feed additive, an osteoporosis-preventing factor (Tanimoto et al. 2001; Yang et al. 2008), humectants, a drug delivery agent (Li et al. 1999; Kishida et al. 1998; Kim et al. 1999; Hashida et al. 1999; Ikumi et al. 2008), a gene vector (Dekie et al. 2000), a curable biological adhesive (Spotniz 1996; Otani et al. 1998; Sekine et al. 2000), a dispersant, and an antibody/enzyme-conjugating (immobilizing) material (Prodhomme et al. 2003; Cheng et al. 2008).

Before applying PGA-based materials to practical use, some issues remain to be solved. The first is cost factor, specifically that the cost estimated under existing

Table 1 Potential applications of poly-γ-glutamic acid and its derivatives (Ashiuchi and Misono 2002b)

Categories	Applications	Details
Biodegradable materials	Thermoplastics, fibers, films	Substitution for chemically synthesized, nonbiodegradable plastics as industrial and daily necessity
	Hydrogels	Substitution for nonbiodegradable water-absorbents such as polyacrylate in diapers; potential application for desert greening
Bioremediation	Flocculants	Substitution for nonbiodegradable flocculants such as polyacrylamide
	Metal absorbents	Removal of heavy metals and radionuclides
Others	Cryoprotectants	Preservation of cryolabile nutrients
	Bitterness-relieving agents	Relief of bitter taste by amino acids, peptides, quinine, caffeine, minerals, etc
	Thickeners	Viscosity enhancement for drinks; prevention of aging of foods such as bakery products and noodles; improvement of textures
	Mineral absorbents	Promotion of absorption of bioavailable minerals such as Ca^{2+}: live stocks, increase in egg-shells strength, decrease in body fat, etc.; human, prevention of osteoporosis
	Humectants	Use for skin-care in cosmetics
	Drug delivers	Improvement of anticancer drugs
	Gene vectors	Use for gene therapy
	Curable biological adhesives	Substitution of fibrin
	Membranes	Separation of heavy metals; enantioselection of amino acids
	Dispersants	Uses in detergents, cosmetics, paper making, etc
	Biomacromolecules-immobilizing materials	

conditions is dozens to hundreds of times higher than that of conventional materials used today. The second is the fact that it is difficult to synthesize artificial PGA by advanced organic chemistry. The most important step now is to elucidate the biosynthetic mechanism of PGA, followed by the construction of a mass-production system. Fortunately, public opinion demands a strong push towards the development of white biotechnology.

2 Occurrence and Producers

Since Kramar (1921) identified *Bacillus anthracis* as the first PGA producer, it has been proven that various organisms, including archaea, bacteria, and animals, can produce PGA (Table 2). Current studies on the molecular physiology of PGA strongly support the fact that it functions as an adaptation agent in various environments (Ashiuchi and Misono 2002b).

Table 2 Structural features of poly-γ-glutamic acid and D-glutamic acid-supplying activity of the producers

Producers	Molecular masses (kDa)	D-Glutamyl unit content (%)	D-Glutamic acid-supplying activity[a]	
			DAT	GLR
Bacillus subtilis (*natto*)	10–1,000	50–80	+[b]	+
Bacillus subtilis subsp. *chungkookjang*	>1,000	35–75	3[c]	36[c]
Bacillus licheniformis ATCC9945	10–1,000	10–100	−[b]	+
Bacillus anthracis	ND[d]	100	−	+
Bacillus megaterium WH320	>1,000	3–10	0[c]	<1[c]
Natrialba aegyptiaca	>1,000	0	0	0
Hydra	3–25	0	ND	ND

[a]*GLR* glutamic acid racemase; *DAT* D-amino acid aminotransferase
[b]+, activity is present; −, activity was absent
[c]mU/mg (of protein); where one unit (U) was defined as the amount of enzyme that catalyzed the formation of 1 μmol of D-glutamic acid from the corresponding substrate(s) per min
[d]ND, not determined

2.1 Poly-γ-DL-Glutamic Acid Producers

Sawamura (1913) isolated a *B. subtilis* strain from a Japanese fermented soybean food known as *natto*. Further studies on its mucilage clarified the existence of DL-PGA (Bovarnick 1942; Kubota et al. 1993; Ashiuchi et al. 2001a).

DL-PGA producers of *Bacillus* were classified into two groups: exogenous glutamic acid-dependent and -independent groups. *B. subtilis* IFO 3335 (Kunioka and Goto 1994; Kunioka 1997), *B. licheniformis* ATCC 9945A (Thorne et al. 1954; Leonard et al. 1958), *B. subtilis* MR-141 (Ogawa et al. 1997), *B. subtilis* subsp. *chungkookjang* (Ashiuchi et al. 2001a), and *B. subtilis* F-2-01 (Kubota et al. 1993) are included in the former category. *B. subtilis* 5E (Shih and Van 2001), *B. subtilis* TAM-4 (Ito et al. 1996), *B. licheniformis* A35 (Cheng et al. 1989), and *B. licheniformis* S173 (Kambourova et al. 2001) belong to the exogenous glutamic acid-independent group. As shown in Table 2, *B. licheniformis* PGA is diverse in its stereochemistry (Pérez-Camero et al. 1999; Thorne and Leonard 1958; Leonard et al. 1958; Cromwick and Gross 1995a). *B. licheniformis* might possess two or more distinct PGA synthetic systems (Cromwick and Gross 1995b). Other than *Bacillus*, some strains of *Staphylococcus epidermidis* also produce DL-PGA for evading mammalian immune defense mechanisms (Kocianova et al. 2005).

2.2 Poly-γ-D-Glutamic Acid Producers

To our knowledge, *B. anthracis* is only a sole D-PGA producer (Table 2). Although D-PGA is avirulent in mammals, it nullifies the immunity of hosts and eventually promotes the severity of anthrax symptoms (Keppie et al. 1963).

2.3 *Poly-γ-L-Glutamic Acid Producers*

L-PGA has been isolated from various extremophilic organisms (Ashiuchi and Misono 2002b). The alkaliphiles *Bacillus halodurans* (Aono 1987) and *Natronococcus occultus* (Niemetz et al. 1997) produce low-molecular-mass L-PGA for neutralization of the near-cell surfaces, whereas the extremely halophilic archaeon *Natrialba aegyptiaca* (Hezayen et al. 2001) produces high-molecular-mass L-PGA (Ashiuchi and Misono 2002b) for the prevention of drastic dehydration under extremely high-saline conditions. A recent study identified salt-inducible L-rich PGA from *Bacillus megaterium* (Shimizu et al. 2007). L-PGA is widely distributed in cnidarians as well, where the polymer is the major constituent of sticky substances in the nematocysts of *Hydra* (Weber 1989, 1990). In cooperation with major bioactive cations like Ca^{2+}, Mg^{2+}, and K^{+}, L-PGA generates and regulates the internal osmotic pressure of such acid producers.

3 Modes of Nonribosomal Amino Acid Polymerization

Antibiotic peptides and polyamino acids often contain nonproteinaceous amino acids (D-glutamyl residues of PGA), unusual linkage formula (γ-amide linkages of PGA), and other unique modifications. They can be synthesized in a ribosome-independent manner. To date, two distinct modes for nonribosomal amino acid polymerization have been proposed (Fig. 1): the thiotemplate mechanism (Kleinkauf and Von Döhren 1996) and by the action of amide ligation (Ashiuchi and Misono 2002b).

The thiotemplate mechanism is catalyzed by nonribosomal peptide synthetases (NRPSs). NRPSs are often called multienzyme systems because of the coexistence of typical adenylation domains, peptidyl carrier domains, specific epimerization

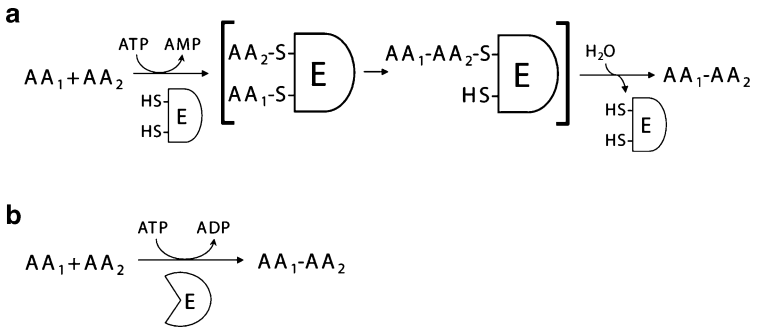

Fig. 1 Nonribosomal amino acid-ligation mechanisms. The thiotemplate (**a**) and amide ligation (**b**) mechanisms are illustrated, where AA_1/AA_2 and E represent the amino acid substrates and enzyme, respectively. In the former, the essential sulfhydryl groups are derived from a moiety of 4′-phosphopanthethein cofactor bound covalently to the seryl residue(s) in a highly conserved region of NRPSs

domains, *N*-methylation domains, and thiolase domains (Vater et al. 1985; Stein et al 1995; Kleinkauf and Von Döhren 1996). They may be characterized by the following observations: (1) the presence of NRPSs consensus sequences; (2) coincident ATP hydrolysis to generate AMP and PPi; (3) production of comparatively small polyamides (3–22 amino acid residues); and (4) strict selectivity for L-amino acid substrates, which sometimes results in a disagreement of the stereochemistry between a polymer produced and the substrate(s). Yamanaka et al. (2008) found that one known polyamino acid, poly-ε-L-lysine, is synthesized via a novel thiotemplate mechanism by a unique NRPS-like enzyme consisting of only a single polypeptide, namely poly-ε-L-lysine synthetase.

The amide ligation mechanism is catalyzed by typical amide ligases, including murein-biosynthetic enzymes (Eveland et al. 1997), or ATP-grasp peptide synthetases, including γ-glutamylcysteine synthetase and D-alanyl–D-alanyl ligase (Galperin and Koonin 1997). These may be characterized via the following observations: (1) presence of consensus sequences typical for amide ligases or sequence similarity to ATP-grasp peptide synthetases; (2) coincident ATP hydrolysis that generates ADP and Pi; and (3) a lack of isomerization activity for amino acid residues in a growing chain, resulting in an agreement of the stereochemistry between a polymer produced and the substrate(s). To our knowledge, D-amino acid residues in nonribosomal peptides generated via the amide ligation mechanism are derived from free D-amino acids in cells. Among the known polyamino acids, cyanophycin (water-insoluble multi-L-arginyl–poly-α-L-apspartic acid) is produced by a probable bifunctional amide ligase, namely cyanophycin synthetase (Aboulmagd et al. 2000, 2001; Hai et al. 2002).

In contrast, due to the extreme instability of *Bacillus* PGA synthetases (Gardner and Troy 1979; Ashiuchi et al. 2001b), attempts were made by means of molecular microbiology to elucidate the synthetic mechanism of PGA (Makino et al. 1989; Kubota et al. 1993; Kunioka and Goto 1994; Cromwick and Gross 1995b; Ashiuchi et al. 2006; Kimura et al. 2009) with little success. The incorporation of D-glutamic acid into bacterial cells in response to excess intracellular D-glutamic acid, and the metabolism of increased D-glutamic acid pools are extremely complicated and the mechanisms by which these occur remain obscure. It is because of these reasons that two different theories regarding PGA biosynthesis have been postulated but remain to be proven.

3.1 Thiotemplate Mechanism for Poly-γ-Glutamate Synthesis

It is indeed known today that *B. licheniformis* produces DL-PGA with various stereochemical compositions (Table 2), but *B. licheniformis* ATCC9945 was thought to produce only D-PGA at that time (Troy 1973b). From the strain, Troy and colleagues found a membrane-associated activity for the synthesis of D-PGA from L-glutamic acid (Troy 1973a; Gardner and Troy 1979). In contrast, D-glutamic acid was neither a substrate nor an inhibitor. The nucleotide thus formed by the

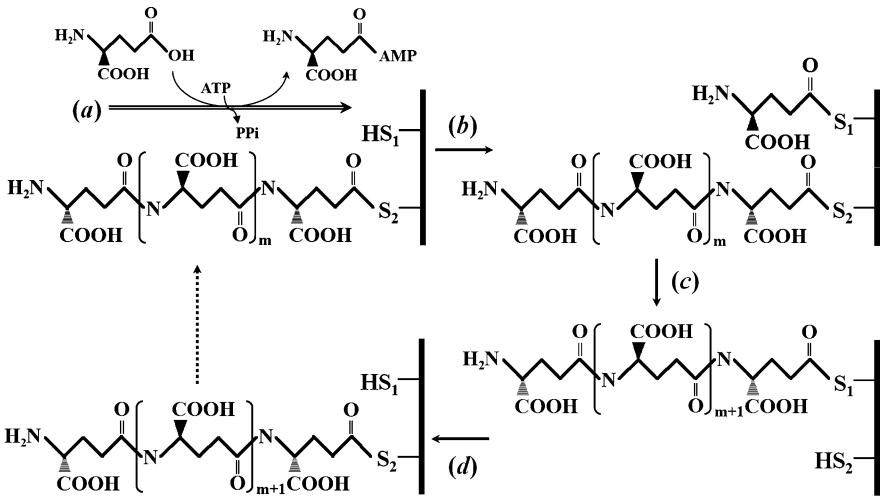

Fig. 2 A proposed thiotemplate mechanism for poly-γ-glutamic acid synthesis. Step *a*, the activation of L-glutamic acid; Step *b*, its covalent binding to the S_1H group of the enzyme and subsequent isomerization into the D-form; Step *c*, the elongation process of a D-PGA chain; and Step *d*, the return of the elongated chain on the S_2H group

by-reaction, i.e. L-glutamic acid-dependent ATP hydrolysis, was AMP (Gardner and Troy 1979).

These findings encouraged a proposed thiotemplate mechanism for the synthesis of PGA (exactly for D-PGA). As shown in Fig. 2, two sulfhydryl groups of enzyme will be involved in catalysis: the loading site S_1H and the elongation site S_2H. First, L-glutamic acid is activated via ATP hydrolysis to generate L-glutamyl-γ-adenylic acid (step *a*), which is covalently bound to an S_1H group and then isomerized into the D-form (step *b*). A thioester bond between a growing D-PGA chain and S_2H suffers nucleophilic attack by the amino group of a D-glutamyl moiety of the loaded S_1H, converting into a new amide bond of the growing chain (step *c*). Finally, the elongated PGA chain returns to S_2H again (step *d*) or may be released in the presence of a reaction terminator. Although information regarding the genome of *B. licheniformis* has already been published (Veith et al. 2004), motifs such as an NRPS-like PGA synthetase remain unidentified. However, if identified, we may be able to determine a novel principle regarding the hyperelongation of biopolyamide molecules and the strict regulation of their stereochemistry.

3.2 Amide Ligation Mechanism for Poly-γ-Glutamate Synthesis

Ashiuchi and colleagues identified a membrane-associated PGA synthetic activity of *B. subtilis* subsp. *chungkookjang* (Ashiuchi et al. 2003a, 2004). As opposed to the case involving *B. licheniformis* ATCC9945, both D- and L-glutamic acid served

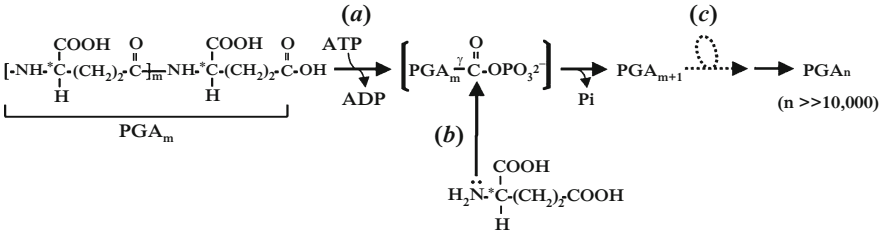

Fig. 3 A proposed amide ligation mechanism for poly-γ-glutamic acid synthesis. Step *a*, the activation of a growing PGA chain; Step *b*, the elongation process of a DL-PGA chain; and Step *c*, the iteration of the reaction steps *a* and *b* in the enzyme

to act as the substrates. The nucleotide formed via coincident ATP hydrolysis was ADP and not AMP (Ashiuchi et al. 2001b; Urushibata et al. 2002a). Some genetic studies indicated that DL-PGA synthetase of *B. subtilis* is not an NRPS-like enzyme but a modular protein complex possessing an amide ligase-like component (Ashiuchi et al. 1999a, 2001b; Urushibata et al. 2002a; Terui et al. 2005). Interestingly, all the molecular machinery for *B. anthracis* D-PGA synthesis (Candela and Fouet 2006), *B. licheniformis* DL-PGA synthesis (Veith et al. 2004), *S. epidermidis* DL-PGA synthesis (Kocianova et al. 2005), and *B. megaterium* L-rich PGA synthesis (Minouchi and Ashiuchi 2009) are homologous to the PGA synthetase of *B. subtilis* in primary structure.

These findings led to the proposed amide ligation mechanism for the synthesis of PGA (Fig. 3). In principle, PGA is not covalently bound to a membrane-associated enzyme at any stage; consequently, an additional anchoring reaction to mureins will be required for the encapsulation of PGA (Candela and Fouet 2005). First, the phosphoryl group of ATP is transferred to the terminal carboxyl group of a growing chain of PGA (Fig. 3, step *a*) and the resulting ADP is liberated from an active site of enzyme. An amide linkage is newly formed via nucleophilic attack of the amino group of either D- or L-glutamic acid monomer on the phosphorylated carboxyl group of the growing chain (step *b*). The polyamide (main) chain of PGA will be highly elongated if a series of the reaction (steps *a* and *b*) iteratively and successively occurs at an active site of enzyme (step *c*). In discussing why such diversity occurs in the molecular sizes and stereochemistry of *Bacillus* PGA (Table 2), the amide ligation mechanism may be somewhat superior to the thiotemplate mechanism.

4 Biosynthesis of Poly-γ-Glutamic Acid Precursors

Although glutamic acid is the precursor of PGA (Ashiuchi and Misono 2002a), it is debatable whether D-glutamic acid serves as a substrate for the synthesis of D- and DL-PGA (Kada et al. 2004; Ashiuchi et al. 2007; Kimura et al. 2009; Fouet 2009). As described above, this is due to the coexistence of two entirely different proposals regarding the PGA synthetic mechanism. Whilst, a recent NRPS study focused on

the synthesis of the D-glutamic acid-containing peptide microcystin and suggested that free D-glutamic acid, synthesized by glutamic acid racemase, is directly incorporated at its corresponding amino acid residue (Sielaff et al. 2003). This fact implies that a unidirectional isomerization of L- to D-glutamic acid residues in a peptide chain cannot be arranged even in the thiotemplate mechanism.

4.1 L-Glutamic Acid Biosynthesis

Endogenous L-glutamic acid in *B. subtilis* is provided via both anabolic and catabolic pathways. Generally, two molecules of L-glutamic acid are synthesized from an L-glutamine molecule and an α-ketoglutaric acid molecule by a coupled system involving L-glutamine synthetase and L-glutamic acid synthase (Belitsky et al. 2000). In the absence of glutamine, L-glutamic acid is synthesized from α-ketoglutaric acid and inorganic ammonia by L-glutamic acid dehydrogenase (Belitsky and Sonenshein 1998). L-Aspartic acid aminotransferase can convert L-aspartic acid and α-ketoglutaric acid into oxaloacetic acid and L-glutamic acid, respectively. Moreover, exogenous amino acids belonging to the glutamic acid family, such as L-arginine and L-proline, are imported and catabolized into L-glutamic acid (Belitsky and Sonenshein 1998; Shih and Van 2001). It is likely that genes responsible for PGA synthesis are induced with excess L-glutamic acid in cells (Urushibata et al. 2002b). Insights into endogenous L-glutamic acid in *B. subtilis* have assisted in characterizing exogenous glutamic acid-independent PGA producers as described above.

4.2 D-Glutamic Acid Biosynthesis

As shown in Table 2, D-amino acid aminotransferase (DAT) and glutamic acid racemase (GLR) are well studied as D-glutamic acid-supplying enzymes of *Bacillus*. More attention is now being paid to the relationship between such enzyme activities and PGA stereochemistry. D- or D-rich PGA producers (*B. anthracis* and *B. licheniformis*) frequently demonstrate a high DAT activity; DL-PGA producers (*B. subtilis*) exhibit GLR activity; and L-rich or L-PGA producers (*B. megaterium* and *N. aegyptiaca*) possess little or no activity for D-glutamic acid supply.

4.2.1 D-Amino Acid Aminotransferase

Usually, DAT converts D-alanine and α-ketoglutaric acid into pyruvic acid and D-glutamic acid with D-alanine provided from the L-isomer by alanine racemase (Ashiuchi and Misono 2002b). Intracellular DAT activity was found in *B. anthracis* (Thorne and Molnar 1955) and *B. licheniformis* ATCC9945a (Thorne et al. 1955).

According to that coupled system, exogenous L-alanine is one of the best substrates for D- or D-rich PGA production. *B. subtilis* subsp. *chungkookjang*, however, did not produce PGA in the presence of L-alanine (Ashiuchi et al. 2001a) despite the fact that the bacterium contains high alanine racemase activity. It seems unlikely that DAT is functionally significant in the synthesis of *B. subtilis* DL-PGA.

4.2.2 Glutamic Acid Racemase

GLR is ubiquitously conserved in bacteria for the purpose of D-glutamic acid synthesis, an essential component of mureins (Doublet et al. 1992) and often called MurI. The expression of MurI is usually downregulated in most bacteria, due to its function as an endogenous DNA gyrase inhibitor (Ashiuchi et al. 2002; Sengupta et al. 2008). Ashiuchi et al. (1998) found exceptionally high levels of GLR activity in a natural strain of *B. subtilis* that can produce PGA in abundance. They purified GLR and cloned the *glr* gene (corresponding to the *racE* gene of domestic *B. subtilis* strains). Due to the absence of GLR function (Ashiuchi et al. 2003b), it is easily overproduced even in *Escherichia coli* cells. In the enzymatic reaction, the velocity of the conversion for L-glutamic acid into the D-isomer is higher than that of the reverse direction (Ashiuchi et al. 1998). The Glr enzyme may have a potential for supplying endogenous D-glutamic acid efficiently. Ashiuchi et al. (1999b) further proved that the *yrpC* gene encodes another GLR of *B. subtilis*. The function of the YrpC isozyme appeared to result in growth inhibition (Ashiuchi et al. 1999b, 2003b), suggesting that it is an MurI-type GLR of *B. subtilis*. In fact, a single disruptant of the *glr* gene of *B. subtilis* required no D-glutamic acid for apparent cell growth, but a double disruptant of the *glr* and *yrpC* genes of *B. subtilis* revealed strict D-glutamic acid auxotrophy (Ashiuchi et al. 2007). It had been suggested that the YrpC isozyme serves as an anaplerotic enzyme for GLR in D-glutamic acid supply. Additionally, Ashiuchi et al. (2007) established a novel phenotype of *B. subtilis*: a conditional D-glutamic acid auxotrophy that happens in the *glr*-single disruptant if *B. subtilis* DL-PGA synthetase was induced enforcedly. The implication of this is that PGA synthetase preferentially consumes endogenous D-glutamic acid.

5 Biosynthesis of Poly-γ-Glutamic Acid

Ashiuchi and colleagues identified the gene cluster responsible for DL-PGA synthesis from the chromosomal DNA of a natural strain of *B. subtilis* (Ashiuchi et al. 1999a, 2001b, 2004). This gene cluster encompassed four open reading frames (ORFs) that are now known as *pgsB*, *-C*, *-A* (or *-AA*), and *-E* (Candela et al. 2005).

Although all the components of *B. subtilis* PGA synthetase (PgsB, -C, -A, and -E) are membrane-associated, some solubilized forms of the Pgs components have been characterized. PgsB was found to catalyze ATP hydrolysis only in the presence of L-glutamic acid (Urushibata et al. 2002a), whereas water-soluble

forms of PgsBC and PgsBCA catalyzed ATP hydrolysis in the presence of either D- or L-glutamic acid (Ashiuchi et al. 2001b). The ATPase-like reaction is required for step *a* in Fig. 3, and the molecular interaction between the Pgs components may be crucial for determining the substrate specificity for a glutamic acid monomer. PGA with a high molecular mass, however, could not be synthesized (Ashiuchi et al. 2003a). The most unique feature of PGA for it to qualify as a macromolecule is that it possesses a molecular mass over 1,000 kDa. In this respect, any water-soluble Pgs components identified to date cannot be defined as PGA synthetases. They are no more than glutamic acid-dependent ATP hydrolases or typical amide ligases that may synthesize a very short γ-glutamyl peptide. For the time being, only a membrane-associated form of PGA synthetase can execute all the steps in Fig. 3 and eventually produce high-molecular-mass PGAs (Ashiuchi et al. 2004).

Further investigation on the structural biology of membranous PGA synthetases should be carried out for further understanding the molecular mechanisms of PGA biosynthesis. The structural features and predicted functions of each Pgs component are summarized here.

5.1 PgsB Component of Poly-γ-Glutamic Acid Synthetase

It is thought that PgsB is the main component in catalysis (Urushibata et al. 2002a; Kimura et al. 2009), as its primary structure resembles those of water-soluble Mg/ATP-dependent amide ligases that catalyze the addition of a short γ-L-glutamyl chain to a folic acid moiety (Eveland et al. 1997; Tomosho et al. 2008).

5.2 PgsC Component of Poly-γ-Glutamic Acid Synthetase

PgsC is likely a membrane-embedded component of the enzyme (Ashiuchi and Misono 2002b). A structural resemblance of PgsC to the *N*-acetyltransferase-domain of *N*-acetylglutamic acid synthetase (Vetting et al. 2005; Min et al. 2009) has been determined. Interestingly, poly-ε-L-lysine synthetase also possesses the tandem repeat of three *N*-acetyltransferase-like domains at the C-terminus and all these domains are important in catalysis (Yamanaka et al. 2008). The role of *N*-acetyltransferase-like architecture(s) is possibly indispensable for the synthesis of *exo*-polyamino acids.

5.3 PgsA Component of Poly-γ-Glutamic Acid Synthetase

PgsA possesses membrane-anchoring regions (Ashiuchi and Misono 2002b) and localizes near cell surfaces (Narita et al. 2006; Lee et al. 2006). Its orthologs can be identified in various organisms, owing to the homology with cytosolic protein

serine/threonine phosphatases where divalent cations, such as Zn^{2+}, Mn^{2+}, Fe^{2+}, and Ca^{2+}, are arranged (Nordlund and Eklund 1995; Rusnak and Mertz 2000). PgsA possibly functions in PGA export and/or a posttranslational modification of D-glutamic acid-containing PGA synthetases of *Bacillus*.

5.4 *PgsE Component of Poly-γ-Glutamic Acid Synthetase*

Compared with other Pgs components, the role of PgsE remains relatively unknown, although a recent publication proposed that it might be functionally identical to a membrane-associated CapE, an essential component of a plasmid-borne PGA synthetic system in *B. anthracis* (Candela et al. 2005). Most recently, Ashiuchi and Yamashiro (2009) accidentally discovered a novel function of PgsE, which may be particularly significant in some plasmid-borne PGA synthetic systems (Ashiuchi et al. 1999a, 2006).

6 Concluding Remarks and Future Perspectives

It has been approximately 100 years since the first PGA producer was identified (Sawamura 1913). Although recent genetic studies have contributed to the identification of the molecular machinery for PGA production (Ashiuchi and Misono 2002a; Candela and Fouet 2006), enzymological analysis is still at an early stage. Considering that relatively small compounds with γ-L- or γ-D-glutamyl residues are formed via an amide ligation mechanism, it is not surprising that PGA can be synthesized in the same manner; however, a chain-elongation process in the synthesis of PGA is peculiar and remains to be elucidated to fully understand the molecular mechanism of PGA biosynthesis. It will be interesting to determine whether the membrane association of PGA synthetase is crucial for the chain-elongation process.

Most plastics and hydrogels today are produced from petrochemicals and are becoming a necessity for present-day industries and daily life due to their convenience and economy. Abuse of these conveniences, however, often gives rise to environmental and health problems (Dearfield and Abernathy 1988). The demands of current society are urgent in developing earth friendly biodegradable macromolecules, whose manufacturing and application will contribute to savings in energy and resources, curbing the greenhouse effect, developing eco-compatible processes and products, and diversifying agriculture for food production. As described in the introduction, PGA is one of these promising macromolecules, and we can find many patents on the improvement of PGA production and its utilization (Ashiuchi and Misono 2002b). The author hopes that the elucidation of the PGA synthetic mechanism leads to the establishment of an efficient manufacturing system of PGA, which is eventually followed by manufacturing of PGA-based eco-materials suitable for future lifestyles.

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Enzymatic Degradation of Poly-Gamma-Glutamic Acid

Keitarou Kimura and Zui Fujimoto

Contents

Abstract Enzymes that degrade poly-γ-glutamate (γ-PGA) are found mainly in bacteria. γ-PGA is a polymer of glutamic acid linked by γ-peptide bond synthesized by a membrane protein complex. It surrounds bacterial cells, and functions as a physical barrier against bacteriophages or phagocytosis and as an extracellular nutrient reservoir. γ-PGA-degrading enzymes of *Bacillus subtilis*, *B. anthracis*, *Flavobacterium polyglutamicum*, *Myrothecium sp.*, and bacteriophages act differently on γ-PGA and they are essential to facilitate or to antagonize such physiological functions of γ-PGA. γ-PGA contains D-isomer of glutamic acid. γ-PGA-degrading enzymes recognize not only the γ-peptide bond but also the stereochemistry of the polymer. Among the γ-PGA-degrading enzymes, *B. anthracis* CapD and

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bacteriophage PghP have been examined in their tertiary structure. Catalytic mechanism and the recognition of stereochemistry of the substrate are discussed based on their 3D structures. Other γ-PGA-degrading enzymes are classified based on the mode of action and substrate specificities. Their catalytic and physiological functions are reviewed.

1 Introduction

Poly-γ-glutamic acid (γ-PGA)-degrading enzymes specifically act on the γ-carboxyl-α-amide bond of a γ-glutamyl polymer, which is resistant to conventional proteases or peptidases. During the early studies on γ-PGA biosynthesis, a γ-PGA-degrading enzyme was used to identify substances synthesized in vitro (Troy 1973a, b). The γ-PGA-degrading enzyme used at that time was not further characterized. However, based on the mode of hydrolytic action, it was presumably an endo-type enzyme similar to CapD of *Bacillus anthracis* (Troy 1973b; Uchida et al. 1993). CapD belongs to the γ-glutamyltranspeptidase (GGT) family (Pfam01019) and is a virulence factor of pathogenic *B. anthracis* that produces γ-PGA; this γ-PGA surrounds the cell as a protective capsule against the host immune system (Candela and Fouet 2005; Uchida et al. 1993). CapD functions both as a hydrolase and transpeptidase, and anchors γ-PGA to the cell wall by its transpeptidase activity.

Another γ-PGA-degrading enzyme of the GGT family is an extracellular enzyme of *B. subtilis* that produces γ-PGA as a nutrient reservoir in the early stationary phase (Kimura et al. 2004b). *B. subtilis* GGT hydrolyzes γ-PGA in the starved late stationary phase to utilize glutamate as a nutrient, which is the concomitant product of γ-PGA hydrolysis. Amino acid sequences of *B. subtilis* GGT and *B. anthracis* CapD are 30% identical. However, these enzymes act differently on γ-PGA and show different cellular localization. *B. subtilis* GGT is an exo-type enzyme, whereas *B. anthracis* CapD is an endo-type enzyme located in the cell envelope (Candela and Fouet 2005; Kimura et al. 2004b). These two enzymes appear to have evolved from a common ancestral protein to perform every physiological function required to survive in the natural environment.

γ-PGA-degrading enzymes can also be found in organisms that do not produce γ-PGA. Fungi and bacteriophages degrade γ-PGA to use it as a nutrient or facilitate infection in γ-PGA-producing host cells (Tanaka et al. 1993a, b; Hongo and Yoshimoto 1970; Kimura and Itoh 2003). Animal macrophage cells appear to possess an enzyme that degrades γ-PGA of *B. anthracis* to protect themselves from the pathogen (Sutherland et al. 2008; Sutherland and Kozel 2009). These enzymes are distinct from the GGT family proteins. Here, we review the enzymatic degradation of γ-PGA with regards to the mode of action, physiological significance, gene expression, substrate specificity, and enzyme structure.

γ-PGA produced by *B. subtilis* is a polymer with stereochemistry. D- and L-glutamates are copolymerized in a single filament (γ-DL-PGA) by the membrane

γ-PGA synthetase PgsBCA (Ashiuchi et al. 1999; Urushibata et al. 2002; Candela and Fouet 2006). In contrast, *B. anthracis* produces γ-PGA consisting solely of D-glutamate (γ-D-PGA) by CapBCAE, which is homologous to PgsBCA (Makino et al. 1988, 1989; Candela et al. 2005). Several models have been proposed to explain how the enantiomers are aligned in γ-DL-PGA based on enzymological, chemical, and physical analyses, but their alignment is still unclear.

As mentioned in other chapters (see chapters, "Pharmaceutical and Medical Applications of Poly-Gamma-Glutamic Acid" and "Food Applications of Poly-Gamma-Glutamic acid" by Akagi et al. and Tanimoto, respectively), γ-DL-PGA of *B. subtilis* is used in food and pharmaceutical applications (Akagi et al. 2005, 2006; Tanimoto et al. 2001, 2007) and γ-D-PGA is a target molecule in anthrax therapeutics (Scorpio et al. 2007). γ-PGA-degrading enzymes are used in some of these application studies. In the last section, structural features of γ-PGA-degrading enzymes are discussed.

2 Occurrence of γ-PGA-Degrading Enzymes

Enzymatic degradation of γ-PGA was first reported in *Flavobacterium polyglutamicum* (Volcani and Margalith 1957) and microorganisms including *B. subtilis*, *B. licheniformis*, *B. anthracis*, and *Myrothecium* sp. are the main source of γ-PGA-degrading enzymes (Throne et al. 1954; Kimura et al. 2004b; King et al. 2000; Uchida et al. 1993; Tanaka et al. 1993a, b) (Table 1). Other than those listed above, there are taxonomically unidentified microbes that have γ-PGA-degrading activity and utilize it as a nutrient (Obst and Steinbüchel 2004). Among them, γ-PGA-degrading enzymes of *B. subtilis* (*natto*) and *B. anthracis* have been extensively investigated because their γ-PGA plays an important role in terms of industrial production or medical treatment, respectively (Candela and Fouet 2005; Scorpio et al. 2007; Richter et al. 2009; Kimura et al. 2004b; Tanimoto et al. 2007; Akagi et al. 2006).

γ-D-PGA injected intravenously into mice accumulates primarily in liver and spleen cells followed by rapid clearance from serum and excretion in urine (Sutherland et al. 2008). The molecular size of γ-D-PGA found in the urine is smaller compared with that of native serum γ-D-PGA. This in vivo degradation of γ-D-PGA occurs at least in spleenic macrophages (Sutherland and Kozel 2009). Macrophage-like J774.2 cells can internalize γ-D-PGA by receptor-mediated endocytosis and degrade it in lysosomes (Sutherland and Kozel 2009). However, lysosomal γ-D-PGA-degrading enzyme has not been identified.

γ-PGA of hydra (the only example of a γ-PGA-producing eukaryote) is relatively small (3–25 kDa) and is an L-isomer (Weber 1990; Szczepanek et al. 2002). Hydra explores nematocyst capsule by increasing the internal osmotic pressure using the γ-PGA. Degradation process or final fate of the γ-PGA synthesized in the nematocyst capsule is obscure.

Table 1 γ-PGA degrading enzymes

Name	Source	Mode of action	Substrate	End products
GGT	*B. subtilis*	Exo-type	γ-DL-PGA γ-D-PGA	DL-glutamate monomer
YwtD	*B. subtilis*	Endo-type (with specificity)	γ-DL-PGA	γ-L-PGA (200–450 kDa) γ-D-PGA (2–5 kDa)
CapD	*B. anthracis*	Endo-type	γ-D-PGA γ-DL-PGA	Oligomer Oligomer
PGA-hydrolase[a]	*F. polyglutamicum*	Endo-type (with specificity)	γ-DL-PGA γ-D-PGA	L-glutamate monomer
PGA-hydrolase[a]	*B. licheniformis*	Endo-type	γ-DL-PGA	Unknown
Poly(γ-glutamic acid) hydrolase	*Myrothecium* sp.	Endo-type (with specificity)	γ-DL-PGA	γ-L-glutamyl tripeptide D-glutamate rich γ-DL-PGA
PghP	Bacteriophage ΦNIT1	Endo-type	γ-DL-PGA	Pentamer, tetramer, trimer
PGA-hydrolase[a]	Bacteriophage NP-1 cl	Endo-type	γ-DL-PGA	Trimer and dimer
PGA-hydroplase[a]	Mammal	Unknown	γ-D-PGA	Unknown
CL-PGA Esterase	*Acremonium* sp.	Unknown	Cross-linked γ-DL-PGA	Unknown

[a]Names are temporally given to these enzymes, because they are not defined in the original articles

γ-Glutamyl hydrolase (also referred to as folate conjugase or pteroyl-γ-glutamyl hydrolase) and glutamate carboxypeptidase II (also referred to as folate hydrolase or pteroylpoly-γ-glutamate carboxypeptidase) hydrolyze the γ-glutamyl linkage in pteroylpoly-γ-glutamate. Its oligomeric glutamate chain is much shorter than that of bacterial γ-PGA and it is not an amino acid homopolymer. These enzymes are outside the scope of this review.

2.1 Bacterial Degradation of γ-PGA

Experimentally confirmed γ-PGA-producing microbes are *B. subtilis*, *B. anthracis*, *B. licheniformis*, *B. thuringensis*, *B. cereus*, *B. pumilus*, *B. amyloliquefaciens*, *B. mojavensis*, *B. atrophaeus*, *B. megaterium*, *Staphylococcus epidermidis*, *Natrialba aegyptiaca*, *Lysinibacillus sphaericus*, and *Fusobacterium nucleatum* (Cachat et al. 2008; Meerak et al. 2008; Kocianova et al. 2005; Candela et al. 2009). With regards to the wide distribution of γ-PGA synthesis among species, γ-PGA-degrading enzymes might also be produced by these bacterial sources. Systematic screening of soil sample implies that γ-PGA-degrading enzymes are distributed widely in taxonomically different bacteria (Obst and Steinbüchel 2004).

2.1.1 *Bacillus subtilis*

The laboratory lineage of *B. subtilis* (*B. subtilis* 168) is a γ-PGA-negative strain. Therefore, *B. subtilis* (*natto*), a starter strain used for "*natto*" fermentation ("*natto*" is a Japanese fermented soybean food) or undomesticated γ-PGA-positive strains have been employed in γ-PGA studies (Stanley and Lazazzera 2005; Ashiuchi et al. 2001; Kimura et al. 2009). γ-PGA-degrading enzymes have been studied mainly in *B. subtilis* (*natto*).

B. subtilis (*natto*) produces γ-DL-PGA in the early stationary phase through regulation by the quorum-sensing system ComQXPA, which governs stationary phase gene expression (Tran et al. 2000; Dubnau 1999; Kimura et al. 2009; Lazazzera 2000; Ogura et al. 2001) (Fig. 1). γ-PGA-degrading GGT expression is also regulated by ComQXPA. ComX, a small peptide secreted in the medium by the ComQ function, binds to the membrane receptor kinase ComP when the ComX concentration becomes high enough for binding. ComP and the cognate response regulator ComA comprise a two-component system. The cell-density signal of ComX is transmitted by phosphorelay from ComP to ComA. ComA is essential for γ-PGA synthesis, but it does not directly regulate the γ-PGA synthetic operon *pgsBCA*. DegQ, which is positively regulated by ComA, and the DegS–DegU two-component system are required for *pgsBCA* expression (Stanley and Lazazzera 2005; Murray et al. 2009; Kimura et al. 2009).

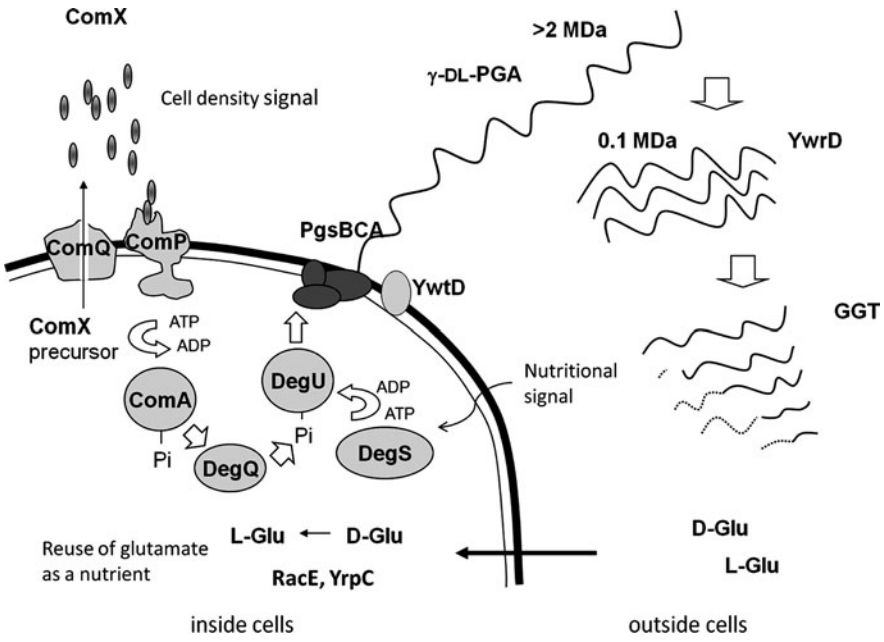

Fig. 1 Schematic representation of the regulatory system of γ-DL-PGA synthesis and degradation in *B. subtilis* (*natto*)

Stationary phase synthesis and degradation of γ-DL-PGA are associated with its physiological role. A high cell density in the stationary phase is a sign of overhanging starvation. It triggers the synthesis of a nutrient reservoir of which plant starch and bacterial polyphosphate are well-known examples. Likewise, *B. subtilis* (*natto*) utilizes γ-DL-PGA as an extracellular nutrient reservoir (Kimura et al. 2004a, b). *B. subtilis* (*natto*) degrades γ-DL-PGA stored outside the cells in the late stationary phase by specific enzymes to reuse glutamate. In the late stationary phase, exoenzymes that enable the utilization of nutritionally unfavorable carbohydrate polymers or proteins are also expressed (Ogura et al. 2003; Tsukahara and Ogura 2008).

Incidentally, γ-DL-PGA degradation in the stationary phase is a well-known phenomenon, and an extracellular GGT was identified as the enzyme responsible for it (Throne et al. 1954; Kunioka and Goto 1994; Ogawa et al. 1991; Abe et al. 1997; Kimura et al. 2004b). Generally, the GGT family of proteins transfer γ-glutamyl compounds to H_2O (in hydrolysis) or amino acids (in transpeptidation) (Tate and Meister 1985; Suzuki et al. 1986; Suzuki and Kumagai 2002; Okada et al. 2006). *B. subtilis* GGT hydrolyzes γ-DL-PGA (V_{max} = 10 μmol/mg protein/min, K_m = 9 μM) and liberates glutamate from the N-terminal end of γ-DL-PGA irrespective of the type of glutamate isomers (Table 2; Kimura et al. 2004b). *6*-Diazo-*5*-oxo-L-norleucine (DON) inhibits both *B. subtilis* and *Escherichia coli* GGT, implying that the catalytic center of both enzymes are structurally redundant, but γ-PGA-hydrolyzing activity is absent in *E. coli* GGT (Table 2; Kimura et al. 2004b). Structural difference and substrate specificity of the GGT family proteins of *B. subtilis*, *E. coli*, and *B. anthracis* are discussed in Sect. 4.1.

B. subtilis (*natto*) NAFM90 (*ggt*::Spc^r), a GGT-deficient mutant, produces γ-DL-PGA as much as wild-type cells. Consequently, NAFM90 (*ggt*::Spc^r) cannot reuse glutamate as the nutrient reservoir. Such an uneconomical overconsumption of food resources does not allow sustainable vegetative growth of cells (Kimura et al. 2004b).

In the late stationary phase, γ-DL-PGA eventually disappears from the culture medium containing wild-type cells. In contrast, the amount of γ-DL-PGA in the NAFM90 (*ggt*::Spc^r) culture medium does not change (Kimura et al. 2004b). However, γ-DL-PGA accumulated in the NAFM90 culture medium has a smaller molecular size (0.1 MDa) than that of the native γ-DL-PGA (>2 MDa) (Kimura

Table 2 Comparison of GGT family proteins of *B. subtilis*, *B. anthracis*, and *E. coli*

	B. subtilis	*B. anthracis*	*E. coli*
K_m to γ-Glu-*p*NA[a]	8 μM	NT[b]	35 μM
Mode of hydrolysis	Exo-type	Endo-type	Exo-type
Cleavage of γ-PGA	Yes	Yes	No
Inhibition by DON[c]	Yes	No	Yes
Loop structure (Pro438[d] to Gly449[d])	No	No	Yes

[a]γ-Glu-*p*NA, γ-glutamyl *p*-nitroanilide, a synthetic chromogenic GGT substrate
[b]*NT* not tested, *B. anthracis* CapD does not hydrolyse γ-Glu-*p*NA
[c]*DON* 6-diazo-5-oxo-noeleucine
[d]Amino acid residue numbers are those of *E. coli* GGT

et al. 2004b). The reduction in size is a result of fragmentation of γ-DL-PGA where *ywrD* gene product is involved. YwrD belongs to the GGT family (amino acid sequence identities to *B. subtilis* GGT and *B. anthracis* CapD are 29% and 26%, respectively). γ-DL-PGA degradation was not observed at all when both *ggt* and *ywrD* were disrupted (Kimura and Itoh, unpublished results). γ-DL-PGA fragmentation by YwrD increases its molar concentration and drastically reduces the viscosity of the medium containing native γ-DL-PGA, which promotes the exotype hydrolysis by GGT (Kimura et al. 2004b). YwrD does not hydrolyze glutathione or γ-Glu-*p*-NA (Minami et al. 2004). YwrD might be an endo-type γ-PGA hydrolase, but we do not have direct experimental evidence about it.

During the late stationary phase when γ-DL-PGA is actively degraded to glutamate monomers by the synergistic reaction of GGT and YwrD, free glutamate concentration in the medium is kept low (<0.03 mM) (Kimura et al. 2004a). *B. subtilis* (*natto*) produces 1–10 mg/ml of γ-DL-PGA in various culture conditions, and the concentration of γ-DL-PGA is equivalent to 8–80 mM of glutamic acid. It seems that glutamate monomers liberated from γ-DL-PGA are rapidly incorporated in the cells and γ-DL-PGA digestion by GGT is the rate-limiting step in glutamic acid consumption. L-Glutamate in the medium severely suppresses GGT expression at the transcription level (Kimura et al. 2004b). This feedback regulation of GGT prevents overconsumption of γ-DL-PGA and enables *B. subtilis* cells to use it for a long time. L-Aspartate, L-asparagine, L-glutamine, and L-proline also partially suppressed GGT expression. Amino acids such as L-isoleucine and L-valine, whose metabolic pathways are not closely related to that of L-glutamate, did not suppress GGT expression at all (Kimura et al., unpublished results).

More than 50% of glutamate in γ-DL-PGA are D-isomers (Nagai et al. 1997). D-amino acids are generally toxic for cells (Soutourina et al. 2000; Yang et al. 2003). *B. subtilis* cells have relatively more abundant glutamate racemase than other bacteria (Ashiuchi et al. 1998; Kada et al. 2004). Glutamate racemases are anabolic enzymes that supply D-glutamate to peptidoglycan in bacteria. However, the glutamate racemases of *B. subtilis* – RacE and YrpC – have both anabolic and catabolic functions (Kimura et al. 2004a). Similar to the cases of catabolic enzymes, RacE and YrpC expression is suppressed in the rich medium, when rapidly proliferating cells need D-glutamate for cell wall construction (Kimura et al. 2004a). Furthermore, D-glutamate clearly exerted a growth-inhibitory effect when racemase genes were disrupted (Kimura et al. 2004a). These observations suggested that in addition to the essential role of supplying D-glutamate for cell wall construction, the glutamate racemases RacE and YrpC act as catabolic enzymes that convert D-glutamate to L-glutamate and are a part of the γ-PGA recycling pathway (Kimura et al. 2004a) (Fig. 1).

B. subtilis and *B. licheniformis* are taxonomically very close to each other. The amino acid sequences of the γ-DL-PGA synthetic *pgsB*, *C*, and *A* gene products are 91%, 91%, and 68% identical when compared with each other; the neighboring genes are also homologous between them (Fig. 2). These two bacteria have essentially the same γ-DL-PGA synthetic and degrading systems. The *ywtD* gene (also referred to as *pgsD* or *pgdS*) is located immediately downstream of the *pgs* operon in

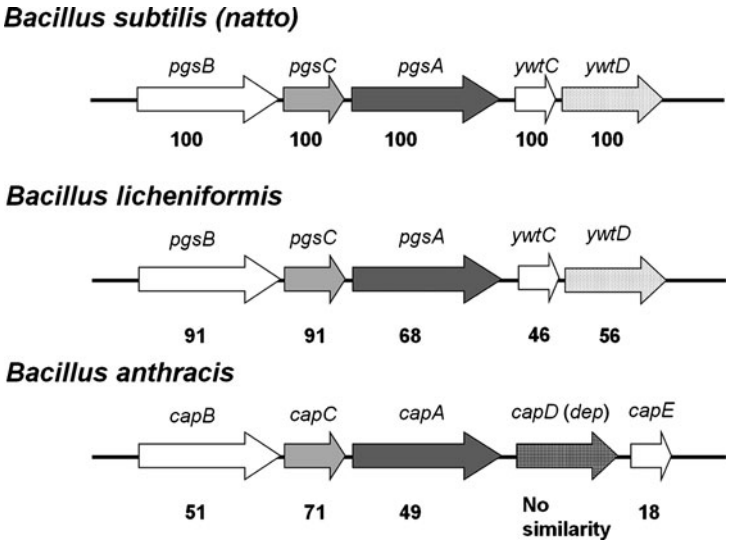

Fig. 2 Structure of the *cap*/*pgs* γ-PGA synthetic operon of *B. subtilis* (*natto*), *B. licheniformis*, and *B. anthracis*. *Numbers* indicate amino acid sequence identities (%) of Cap/Pgs proteins to those of *Bacillus subtilis* (*natto*). The *capD* gene of *B. anthracis* is similar to *ggt* and *ywrD* genes of *B. subtilis* or *B. licheniformis*. *ggt* and *ywrD* genes reside in loci far from the *pgs* region in these two bacteria. *B. anthracis* does not possess the *ywtD* gene

B. subtilis and *B. licheniformis*. Recombinant YwtD hydrolyzes γ-DL-PGA in vitro into large (200–450 kDa) and small (2–5 kDa) fragments (Chunhachart et al. 2006; Ashiuchi et al. 2006; Suzuki and Tahara 2003). The large fragments are L-glutamate-rich polypeptides and the small fragments are D-glutamate rich. Analysis of terminal glutamate of the fragments produced by YwtD implies that YwtD hydrolyzes the γ-glutamyl linkage between D-glutamates (Ashiuchi et al. 2006). The recombinant YwtD hydrolyzes γ-DL-PGA but not synthetic oligo-γ-glutamate (Chunhachart et al. 2006). YwtD probably recognizes the long chain of γ-DL-PGA. *B. subtilis* (*natto*) NAFM61 (*ggt*::Spc^r, *ywrD*::Erm^r) does not degrade γ-DL-PGA at all (see above), but the large and the small fragment corresponding to the in vitro hydrolysis products by YwtD were not found in the culture medium. Therefore, the in vivo significance of YwtD is obscure. The absence of *ywtD* in *B. anthracis*, whose γ-D-PGA is tightly anchored to the cell wall (Candela and Fouet 2005; Richter et al. 2009), prompts us to hypothesize that YwtD is involved in releasing γ-PGA to the culture media.

2.1.2 *Bacillus anthracis*

γ-D-PGA of *B. anthracis* covers cells as a protective capsule. Strains lacking γ-D-PGA are avirulent because they cannot escape the phagocytic attack of the host immune system (Fouet and Mock 2006). Therefore, γ-D-PGA is a target molecule in anthrax therapeutics (Scorpio et al. 2007; Richter et al. 2009). The *capD* gene of

B. anthracis is a part of the γ-D-PGA synthetic operon (Fig. 2). CapD is not essential for γ-D-PGA synthesis, but is required for attaching the synthesized glutamate filament to cell wall peptidoglycan (Candela and Fouet 2005; Richter et al. 2009). γ-D-PGA released in the medium as a free form in the absence of CapD has no such barrier function (Candela and Fouet 2005). CapD is located on the cell envelope, whereas the GGT of *B. subtilis* is secreted in the medium.

CapD belongs to the GGT family. GGT, as its name indicates, is an enzyme that hydrolyzes or transfers a wide variety of γ-glutamyl moiety-containing substrates such as glutathione (Tate and Meister 1981). However, unlike the GGT of *B. subtilis,* which is an exo-γ-glutamyl hydrolase that liberates a glutamate monomer from the N-terminal end of γ-PGA, CapD performs an endo-type hydrolysis (Uchida et al. 1993; Candela and Fouet 2005; Richter et al. 2009). CapD does not hydrolyze γ-glutamyl-*p*-nitroanilide (γ-Glu-*p*-NA), a popular substrate of the GGT family proteins (Tate and Meister 1985). DON, which inhibits most GGTs including those of *B. subtilis* or *E. coli* has no effect on CapD (Richter et al. 2009; Table 2).

Substances that inhibit CapD but not mammalian GGT are candidates for anti-anthrax drugs. A compound that inhibits CapD was found recently (Richter et al. 2009). The inhibitory compound, capsidin, which mimics the backbone structure of γ-D-Glu–D-Glu, inhibits CapD with an IC_{50} value of 6.63 μM and prevents the capsulation in vivo. Capsidin noncompetitively inhibits CapD by acetylation of the active site threonine without affecting mammalian GGT (Richter et al. 2009).

B. anthracis lacks *ywtD* gene, which resides at the corresponding position of CapD in the genomes of *B. subtilis* and *B. licheniformis*. In *B. subtilis* and *B. licheniformis*, γ-DL-PGA is secreted in the medium as a free form (Kunioka and Goto 1994; Kimura et al. 2004b; Birrer et al. 1994). YwtD might cleave γ-DL-PGA once anchored to the cell wall during synthesis. The in vitro hydrolytic activity of YwtD on γ-DL-PGA supports this hypothesis. Experimental elucidation is awaited.

2.1.3 Other Microorganisms

F. polyglutamicum utilizes γ-DL-PGA as a sole carbon and nitrogen source (Volcani and Margalith 1957). γ-DL-PGA-hydrolyzing activity was observed in the cell extracts of *F. polyglutamicum*. L-Glutamic acid was the only enzymatic breakdown product and its amount was equivalent to that in the substrate (Volcani and Margalith 1957). The *F. polyglutamicum* enzyme appears to cleave the γ-L-glutamyl–L-glutamate linkage in γ-DL-PGA because the γ-DL-PGA filament is a copolymer of L- and D-glutamates (see Sect. 3). This enzyme, which is insensitive to iodoacetate and capable of hydrolyzing pteroyl-γ-glutamic acid, is distinct from the other γ-PGA-degrading enzymes. Volcani and Margalith also reported that they obtained several different bacteria utilizing γ-DL-PGA as a sole source of nitrogen and carbon in soil samples, but only *F. polyglutamicum* was characterized.

Birrer et al. reported cell-associated γ-DL-PGA degradation in *B. licheniformis* and King et al. reported γ-PGA-associated γ-DL-PGA degradation in *B. licheniformis* ATCC9945a (Birrer et al. 1994; King et al. 2000). The γ-PGA-associated

enzyme is an endo-type that does not hydrolyze the synthetic dipeptide thioester substrate AcNH-D-Glu-γ –D-Glu–γ-SEt. In both cases, enzymes were not purified or characterized in detail.

Tanaka et al. screened 683 fungal strains by monitoring the reduction in the viscosity of γ-DL-PGA to find endo-type γ-DL-PGA-degrading enzymes (exo-type hydrolysis does not drastically reduce viscosity). The 68 kDa γ-PGA hydrolase was homogeneously purified from *Myrothecium* sp. TM-4222 culture broth (Tanaka et al. 1993a, b). It degrades γ-DL-PGA to mixture of oligo-γ-L-glutamate. The major hydrolytic product was γ-L-glutamyl tri-peptide, but no L-glutamate monomer was observed. The digestion of γ-DL-PGA yields a D-glutamate-rich fragment (D-Glu:L-Glu = 89:11) (Tanaka et al. 1993a, b). This D/L ratio is quite high compared to the ratio in the substrate (D:L = 55:45) (see Sect. 3.1).

2.2 *Bacteriophage-Related γ-PGA Degradation*

Contamination by bacteriophages continues to be a problem in the fermentation industry. Bacteriophages that spoil *natto* fermentation were collected to survey the damage (Nagai and Yamasaki 2009). They were detected mainly from *natto*, but were found also on the surface of drains, containers, and in pipes of *natto* factories.

For suppliers of *natto*, phage contamination that spoils the growth of host cells or stops the fermentation process is easy to handle. They can detect contamination visually and can discard spoiled products before shipping. However, if the number of contaminating bacteriophages is small enough (usually below 10 phages/package) to allow apparently normal growth of *B. subtilis* (*natto*), the contaminated product escapes monitoring. In such a case, suppliers receive an unfavorable response from consumers who purchased them due to a γ-PGA degrading enzyme produced by the phage (see below).

2.2.1 PghP of *B. subtilis* (*natto*) Phage ΦNIT1

Bacteriophage-related γ-PGA degradation has been known since the 1970s (Hongo and Yoshimoto 1970). Strong γ-DL-PGA-degrading activity was found in the *B. subtilis* (*natto*) culture supernatant infected with ΦNIT1 isolated from spoiled *natto*, and PghP (poly-γ-glutamate hydrolase P) was homogeneously purified (Kimura and Itoh 2003). This enzyme degrades γ-DL-PGA to γ-glutamyl oligomers and finally to γ-glutamyl penta-, tetra-, and tri-peptides. PghP is a monoiodoacetate-sensitive 25 kDa metallopeptidase requiring Zn^{2+} or Mn^{2+} for hydrolysis (Kimura and Itoh 2003).

PghP hydrolyzes γ-DL-PGA and very quickly decreases polymer viscosity. PghP (1 mg) can hydrolyze 1.5 g of γ-DL-PGA to the final products within 1 min (Kimura and Itoh 2003). *Natto* is mixed with a soy sauce or other seasonings and stirred just

before serving to give the essential sticky texture to it, which allows contaminated PghP to act and eliminate viscosity.

2.2.2 Effect of γ-PGA Degradation on Bacteriophages

B. subtilis (*natto*) colonies are covered by a sticky material produced in large amounts (Fig. 3). The sticky material is a mixture of γ-DL-PGA and levan (contribution of levan to the stickiness and viscosity is small because disruption of the levan synthetic gene minimally affects stickiness and viscosity). Breakdown of the sticky material by PghP allows phage progenies to easily spread and infect host cells (Kimura and Itoh 2003). *E. coli* phage 29 has an endo-*N*-acetylneuraminidase in its phage particle (spike) for hydrolyzing the K1 capsule (poly-α-2,8-linked sialosyl unit) (Kwiatkowski et al. 1982). Some phages of *Klebsiella* and *Streptococcus* also have enzymes for hydrolyzing capsular polysaccharides on phage particles (Cescutti and Paoletti 1994). PghP is not displayed on the phage particle. Thus, the first phage cannot remove γ-DL-PGA for infection (the ΦNIT1 receptor is not identified, but phages have to establish contact with the host cell surface). PghP synthesis during phage propagation in host cells appears to be antithetical to its function. However, *B. subtilis* γ-DL-PGA is produced in the stationary phase (Tran et al. 2000; Kimura et al. 2009). Phages can infect young colonies without the barrier of γ-DL-PGA in a natural environment, and the PghP produced can spread around to hydrolyze γ-DL-PGA of neighboring colonies. PghP expression on the phage particle might be a less effective strategy in this case. γ-DL-PGA is produced in a large amount and usually exists as a viscous water absorbent surrounding cells (Fig. 3). Therefore, allowing the degrading enzyme to spread as much as possible by the pioneer phage might be a good group strategy.

The host cell range for ΦNIT was examined using 49 *B. subtilis* test strains (Ackermann et al. 1995). ΦNIT1 propagates and makes a clear halo on grasses of *B. subtilis* cells including strains HER1395, HER1313, SIM46, HSY20, Q:1:1.3, IP005, SIM218, and IP032 (Kimura et al., unpublished results). The host specificity spectrum was different from any other typing phages reported previously (Ackermann et al. 1995).

The *pghP* gene (accession no. AB091475) in the phage genome has no significant homology to known enzymes. It appears to be transcribed together with the upstream gene encoding a hypothetical pectin lyase-like protein (Fig. 4). The pectin

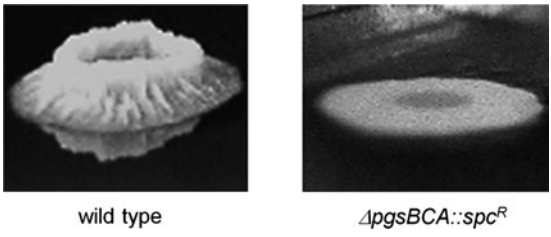

Fig. 3 Morphology of *B. subtilis* (*natto*) colonies. Wild-type and γ-DL-PGA-deficient mutant (*pgsBCA*::Spc^R) cells are grown on GSP agar plates (Nagai et al. 1997) overnight

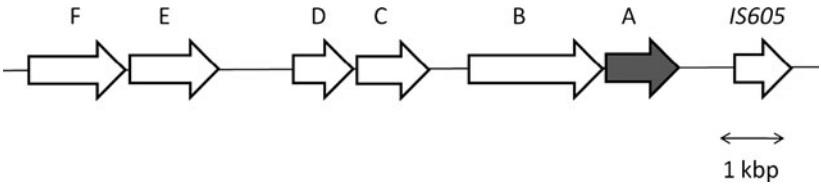

Fig. 4 Schematic illustration of *pghP* and its neighboring genes in the bacteriophage ΦNIT1 genome. PghP coding region (*shaded*) and possible ORFs are shown by *arrows*. A, PghP; B, pectin lyase-like protein; C, hypothetical protein; D, dihydrofolate reductase; E, adenylate kinase; F, thimidylate synthase

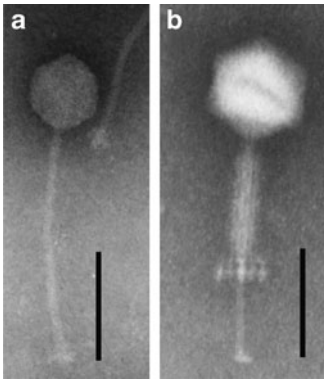

Fig. 5 Electron micrographs of *B. subtilis* (*natto*) bacteriophages JNDMP (**a**) and ONPA (**b**). *Bar* = 100 nm. Adapted from Nagai and Yamasaki (2009) with publisher's permission. Phage particles are negatively stained with 2% phosphotungstic acid and observed under an electron microscope (JEOL-1200 EX, Japan Electronic Optics Laboratory, Tokyo, Japan)

lyase-like protein might be involved in digesting cell wall components during phage propagation.

2.2.3 PghP Distribution

Bacteriophages isolated from spoiled *natto* were classified into two groups (groups I and II) based on genome structure and phage particle morphology (Nagai and Yamasaki 2009) (Figs. 5, 6). The ΦNIT1 phage belongs to group II, having a hexagonal head and sheathed tail. The *pghP* gene was found in both types of phage (Kimura et al., unpublished results). A mobile insertion sequence of the *IS605* family was found in the 3′-flanking region of *pghP* (Fig. 4). *pghP* was possibly distributed by horizontal movement from one phage genome to another.

The γ-DL-PGA-degrading enzyme of bacteriophage NP-1 cl produces trimers and dimers of glutamic acid as the final products (Hongo and Yoshimoto 1970). Furthermore, phages producing γ-DL-PGA-degrading enzyme but not possessing genes homologous to *pghP* were found in soil (Kimura and Itoh 2003; Kimura et al., unpublished results). A variety of γ-PGA-degrading enzymes is suggested in bacteriophages.

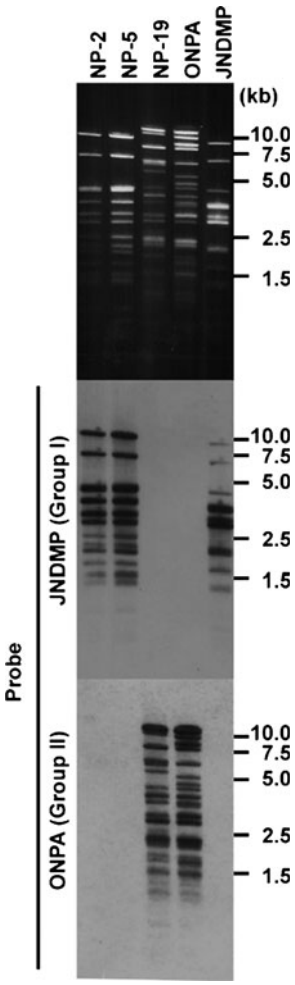

Fig. 6 Southern hybridization analyses of *B. subtilis* (*natto*) phage genomic DNA. Phage genomic DNA is digested with *Hin*dIII. Total genome DNA of phage JNDMP or ONPA is labeled and used as a probe. Adapted from Nagai and Yamasaki (2009) with publisher's permission

3 Stereochemistry of γ-PGA and Substrate Specificity

γ-PGA-degrading enzymes act differently on γ-D-PGA and γ-DL-PGA. For example, CapD can digest both substrates, while PghP poorly hydrolyzes γ-D-PGA (Scorpio et al. 2007). γ-D-PGA forms a left-handed helix and γ-DL-PGA is thought to form a helix or a flexible β-sheet/coil structure depending on pH, ionic strength, and concentration (Zanuy and Alemán 2001; Zanuy et al. 1998; He et al. 2000; Saito et al. 1974). In γ-DL-PGA, sequence distribution of glutamate enantiomers affects its microstructure (de Ilarduya et al. 2002). This section reviews recent studies on γ-PGA structure with regards to substrate specificity of the γ-PGA-degrading enzymes.

Chemical or physical treatment can alter the higher-order structure of γ-PGA. For example, γ-ray irradiation induces cross-linking between γ-DL-PGA filaments (Matsui et al. 2008). Grafting hydrophobic side chains such as phenylalanine causes γ-DL-PGA to self-assemble and form nanoparticles (Akagi et al. 2005, 2006). These modified γ-PGAs are used for various industrial purposes (Yoshikawa et al. 2008; Buescher and Margaritis 2007). Enzymatic digestion of modified γ-PGAs is mentioned below.

3.1 Stereochemistry of γ-PGA

Chemical, enzymological, and physical analyses suggest that γ-DL-PGA produced by *B. subtilis* is a copolymer consisting of D- and L-glutamate incorporated into a single filament, although we cannot exclude the possibility that small amounts of D- or L- glutamate homopolymers are also produced.

Enantiomer composition and sequence distribution of γ-DL-PGA was first explored by Tanaka et al. (1993a) using PGA hydrolase isolated from the *Myrothecium* sp. TM-4222 culture supernatant . In this study, the random distribution of isomers in γ-DL-PGA was not considered and heterogeneous isomer units were suggested (Tanaka et al. 1993a, b). Likewise, γ-DL-PGA microstructure in which D- or L-glutamate repeating units are alternately linked in a single chain is proposed by chemical hydrolysis of γ-DL-PGA (Wang et al. 2008). Wang et al. chemically hydrolyzed γ-DL-PGA (50% D-Glu content) and obtained γ-glutamyl dipeptides. The γ-glutamyl dipeptides D–D, L–L, D–L, and L–D were separated by chiral-specific HPLC and these four dipeptides appeared in a ratio of 5.9:6.0:1.0:1.0. If the enantiomers are randomly linked in a single chain, this ratio would be close to 1.0:1.0:1.0:1.0. Therefore, there seem to be blocks of homopolymers in the filaments (Wang et al. 2008).

PghP digests γ-DL-PGA and produces pentamers, tetramers, and trimers of glutamic acid, but it cannot hydrolyze synthetic hexa-oligo-γ-D-glutamate (Shiga and Kimura, unpublished results). The stretch of D-glutamate in γ-DL-PGA is perhaps less than a hexamer. However, estimation of the stereochemical structure of γ-DL-PGA only from enzymatically digested products should be performed carefully. Generally, peptide chains longer than a hexamer are required to form a helical structure (Moretto et al. 2008). PghP might randomly cut the γ-glutamyl peptide bond regardless of the stereochemistry of the cutting site, and a structural change in the substrate such as a shift from helix to random coil might prevent the enzyme from recognizing the shortened substrate.

^{13}C NMR analyses of γ-CH and β-CH_2 in α-esterified γ-DL-PGA (poly α-ethyl γ-DL-glutamic acid) and various synthetic γ-PGA reference molecules can detect the local sequence of enantiomers. de Ilarduya et al. showed that γ-DL-PGA consists of a block stereocopolymer of D and L units as well as a minor amount (about 10%) of two enantiomerically pure homopolymers, which is consistent with the microstructure proposed by enzymatic and chemical cleavage experiments

(de Ilarduya et al. 2002; Wang et al. 2008; Tanaka et al. 1993b; Ashiuchi et al. 2006; Chunhachart et al. 2006).

3.2 Mode of Action in γ-PGA Hydrolysis

There exist three types of hydrolysis: exo-type, nonspecific endo-type, and endo-type. GGT liberates glutamate from the N-terminal end of the polypeptide and demonstrates no isomer specificity (Kimura et al. 2004b). PghP hydrolyzes γ-DL-PGA by the endo-type mechanism (Kimura and Itoh 2003; Scorpio et al. 2007). *B. anthracis* CapD hydrolyzes both γ-D-PGA and γ-DL-PGA by the endo-type mechanism (Scorpio et al. 2007). γ-D-PGA is covalently attached to the cell wall by the transpeptidase activity of CapD. CapD hydrolyzes "native" γ-D-PGA produced as a free form in the culture medium of the *capD* mutant strain RTC40 (*capD*::Spc^r), as well as "denatured" γ-D-PGA isolated from wild type cells by autoclave (Kimura and Fouet, unpublished results). The final product of CapD hydrolysis has not been examined in detail.

YwtD of *B. subtilis* and γ-PGA hydrolases of *F. polyglutamicum* and *Myrothecium* sp. are endo-type hydrolases with stereochemical specificity (Ashiuchi et al. 2006; Volcani and Margalith 1957; Tanaka et al. 1993a). These enzymes produce γ-L-PGA or γ-D-PGA from γ-DL-PGA. YwtD does not hydrolyze synthetic oligo-γ-glutamate (octamer). It probably recognizes the cleavage site of peptide chains longer than eight residues (Chunhachart et al. 2006).

3.3 Modified γ-PGA

Irradiating γ-DL-PGA induces random cross-linking between the filaments of the glutamate chain. The cross-linked γ-DL-PGA (CL-PGA) irradiated by 20 kGy γ-ray absorbs 4,500% (w/w) of water and forms a hydrogel with various properties (Hara 2001). CL-PGA degradation was examined in compost to assess its biodegradability by measuring amounts of CO_2 released based on the ISO14855 standard method. Eighty percent of the CL-PGA was degraded within 2 weeks (Hara 2001). Matsui et al. found a fungal strain, FK-1, belonging to the genus *Acremonium* that produces a CL-PGA liquefying enzyme (Matsui et al. 2008). It also hydrolyzes γ-DL-PGA chemically cross-linked by an ester bond (Matsui et al. 2008). The liquefying enzyme has not been further characterized.

Amphiphilic poly(α-alkyl γ-glutamate) such as γ-DL-PGA derivatives conjugated with L-phenylalanine ethylester assembles into nanoparticles in water (Akagi et al. 2005, 2006). GGT of *B. subtilis* is known to degrade the backbone of the particle, which is a promising material as a drug delivery carrier (Yoshikawa et al. 2008).

4 Structural Analyses of γ-PGA-Degrading Enzymes

Among the GGT family proteins, the crystal structure of *E. coli* GGT in complex with L-glutamate was the first to be determined (Okada et al. 2006). The *E. coli* GGT structure is helpful for speculating the structure, substrate recognition, and catalytic mechanisms of *B. subtilis* GGT and *B. anthracis* CapD because *E. coli* GGT cannot hydrolyze γ-PGA even though these three proteins are homologous in their amino acid sequences (Fig. 7). The crystal structure of CapD was determined recently (Wu et al. 2009). The structural analysis of PghP is also underway (Fujimoto et al. 2009).

4.1 CapD of B. anthracis *and GGT of* B. subtilis

The GGT family of proteins is a heterodimer enzyme generated from a single polypeptide precursor by posttranslational autocatalytic process (Suzuki and Kumagai 2002). The crystal structure of GGT from *E. coli* K-12 was determined in complex with a γ-glutamyl-enzyme intermediate or L-glutamate (Okada et al. 2006). Successive structural analysis of the precursor protein clarified the maturation mechanism of the enzyme (Okada et al. 2007). GGT has a stacked αββα fold,

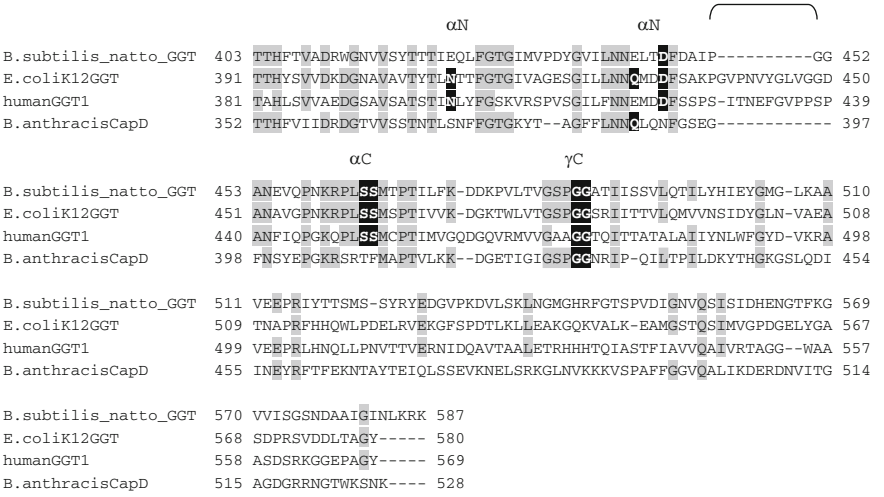

Fig. 7 Amino acid sequence alignment of the small subunit of GGT family proteins from *B. subtilis* (*natto*), *E. coli,* human, and *B. anthracis*. Amino acid residues involved in substrate binding in *E. coli* GGT are shown in *white bold*, and moieties recognized by them are shown as αN (α-amino group), αC (α-carboxyl group), and γC (γ-glutamyl carbonyl oxygen) (Okada et al. 2006). Amino acid sequence corresponding to the loop structure (Pro438–Gly449) covering the groove leading to the substrate pocket is *bracketed*. The sequence alignment is created by CLUSTAL W (Thompson et al. 1994)

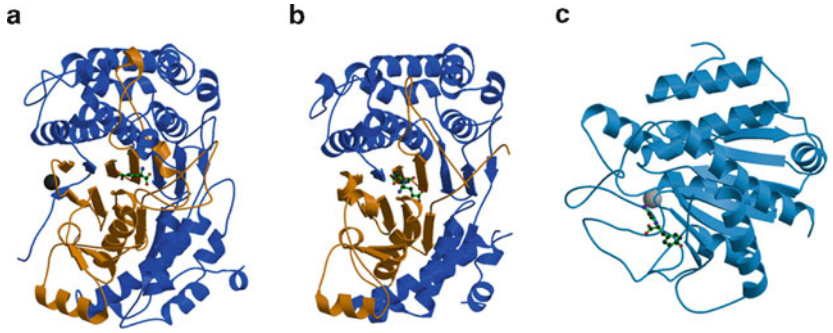

Fig. 8 Ribbon drawings of the crystal structures of *E. coli* GGT (protein bank code 2DBX) (**a**), *B. anthracis* CapD (3GA9) (**b**), and bovine CPA (3CPA) (**c**). The bound ligands (L-glutamate, L-glutamyl-L-glutamate, and glycyl-L-tyrosine) are shown in *green ball-and-stick drawings*. The large and small subunits of GGT and CapD are shown in *blue* and *orange*, respectively. The figure is prepared by Raster3D (Merritt and Bacon 1997)

similar to the folds seen in members of the N-terminal nucleophile hydrolase superfamily (Fig. 8). The small subunit is surrounded by the large subunit and β-strands of the large and small subunits form the two central β-sheets. The γ-glutamyl moiety of the intermediate covalently binds to Thr391, which is the N-terminal residue of the small subunit and the catalytic residue of the enzyme. The bound L-glutamate in the enzyme pocket interacts with Arg114, Ser462, Ser463, Gln430, Asp433, Gly483, and Gly484 by hydrogen bonds and salt bridges. With the exception of Arg114, all residues involved in substrate binding are located in the small subunit (Fig. 7). The α-carboxyl group of the γ-glutamyl moiety is bound to Arg114, Ser462, Ser463, and the α-amino group is bound to Asn411, Gln430, and Asp433. The γ-glutamyl carbonyl oxygen atom is hydrogen-bonded with main-chain amino groups of Gly483 and Gly484.

Amino acid residues corresponding to Arg114, Gly483, and Gly484 of *E. coli* are conserved in *B. subtilis* GGT and *B. anthracis* CapD (Fig. 7). Ser462, Ser463, and Asp433 are conserved in *B. subtilis* GGT but not in *B. anthracis* CapD. Neither Gln430 nor Asn411 are conserved. It is noteworthy that the loop structure from Pro438 to Gly449 of *E. coli* GGT that covers the groove leading to the binding pocket is missing in *B. subtilis* GGT and *B. anthracis* CapD (Fig. 7). The loop structure contains a Tyr444 residue forming a wall that shields the pocket from the solvent and probably prevents large molecules such as γ-PGA from accessing the catalytic site in *E. coli* GGT (Okada et al. 2006). Mammalian GGT that cannot hydrolyze γ-PGA has the loop structure similar to that of *E. coli* GGT. The substrate-binding pockets of bacilli GGTs are more open than those of other GGTs.

Neither *B. subtilis* GGT nor *B. anthracis* CapD displays stereochemical specificity in the enzyme reaction. This might be explained by the absence of residues corresponding to Asn411 and Gln430 that bind to the α-amino group of the substrate. Ser463 and Ser464 bonded with the α-carboxy group in *E. coli* GGT

are conserved in *B. subtilis* GGT but not in *B. anthracis* CapD. *B. subtilis* GGT probably recognizes the N-terminal end of the polypeptide by Gly483, Gly484, Ser463, and Ser464 residues that bind the α-carboxyl group and γ-glutamyl carbonyl oxygen. CapD seems to recognize neither α-amino nor α-carboxyl groups at the N-terminal end of γ-PGA. CapD probably interacts only with the γ-glutamyl carbonyl oxygen of γ-PGA via the conserved Gly–Gly motif and degrades it by an endo-type mechanism without stereochemical specificity. The crystal structure of CapD was recently determined in complex with a nonhydrolyzable analog, α-L-Glu–L-Glu (Wu et al. 2009). Its overall structure resembles that of *E. coli* GGT. Substrate recognition by residues Pro427–Gly428–Gly429 that appeared to contribute to the CapD hydrolytic reaction was structurally confirmed.

4.2 PghP of Bacteriophage ΦNIT1

PghP and CapD are endo-type hydrolases. However, as discussed above, PghP – a metalloenzyme – does not belong to the GGT family and is an orphan enzyme. BLAST search of PghP retrieved genes from *Bacillus* phage SPP1, *B. pumilus*, *B. licheniformis, B. subtilis, Staphylococcus haemolyticus,* and *B. cereus*. These are all hypothetical proteins.

PghP of bacteriophage ΦNIT1 was crystallized and its structural analysis is in progress (Fujimoto et al. 2009). Preliminary analyses revealed that PghP is a globular protein having an open α/β mixed-core structure with a seven-stranded parallel/antiparallel β-sheet. The overall core structure resembles that of bovine carboxypeptidase A (CPA; Fig. 8) or related zinc peptidases, which are phylogenetically very far from PghP and belong to the M14 metallopeptidase family (Kilshtain-Vardi et al. 2003; Cappalonga et al. 1992; MEROPS: http://merops.sanger.ac.uk). The CPA crystal structure was first determined by Lipscomb et al. (1970) and successive structural analyses revealed the catalytic residues and substrate recognition mechanism in detail. CPA has one zinc ion at the catalytic center coordinated by two histidine and one glutamate residues, and the bound zinc ion and another glutamic acid, Glu270, are responsible for catalysis. The His–Glu–His zinc coordinate motif of CPA was found also in PghP at a topologically equivalent position (Fujimoto and Kimura, manuscript in preparation). Structural comparison of PghP, M14 metallopeptidases, and the GGT family proteins can be helpful for elucidating the molecular mechanisms of the γ-PGA hydrolyzing enzymes, as well as the recognition mechanism of γ-PGA stereochemistry.

5 Conclusion and Future Perspectives

B. subtilis, *B. anthracis*, and other microbes produce γ-PGA having different physiological functions and utilize them to survive in natural environments. As observed in the GGT family proteins of *B. anthracis*, *B. subtilis*, and *E. coli*,

degradation enzymes appear to have evolved to perform such functions. Although γ-PGA production is not examined directly, genes homologous to the *pgs*/*cap* γ-PGA synthetic system can be found in genomes of *Oceanobacillus iheyensis* HTE831, *Desulfitobacterium hafniense* Y51, *Francisella tularensis* Schu 4, *Leptospira interrogans serovar lai 56601*, and several other microorganisms (in some cases, *pgsA*/*capA* homologue is missing) (http://www.ncbi.nlm.nih.gov/). These bacteria are potential sources of novel γ-PGA-degrading enzymes.

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Pharmaceutical and Medical Applications of Poly-Gamma-Glutamic Acid

Takami Akagi, Michiya Matsusaki, and Mitsuru Akashi

Contents

Abstract Poly(amino acid)s have received considerable attention for biomedical applications. Poly(γ-glutamic acid) (γ-PGA), a natural polymer, is synthesized by several gram-positive bacteria. γ-PGA is anionic, water soluble, biodegradable, edible, nontoxic, and nonimmunogenic for humans and the environment, and its α-carboxylate side chains can be chemically modified to introduce various drugs, or to modulate the amphiphilicity of the polymer. These features of γ-PGA are very useful for pharmaceutical and biomedical applications. This paper reviews the preparation of polymeric drugs, nanoparticles, and hydrogels composed of γ-PGA and their medical applications as drug carriers and tissue-engineering materials. γ-PGA–drug conjugates, nanoparticles, and hydrogels fabricated from γ-PGA or its derivatives have wide application for drug delivery system and regenerative medical technique.

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1 Introduction

γ-PGA is a naturally occurring poly(amino acid) that is synthesized by certain strains of *Bacillus* (Kubota et al. 1993). The polymer is made of D- and L-glutamic acid units linked through the α-amino and the γ-carboxylic acid groups, respectively, and its α-carboxylate side chains can be chemically modified to introduce various bioactive ligands, or to modulate the overall function of the polymer (King et al. 1998; Morillo et al. 2001; Prodhomme et al. 2003; Tachibana et al. 2003; Shimokuri et al. 2004). With the exception of biologically synthesized γ-PGA, chemically synthesized γ-PGA has been reported. γ-PGA cannot be synthesized by polycondensation of glutamic acid, because intramolecular cyclization predominantly proceeds to form a stable five-membered lactam, pyroglutamic acid. Moreover, it is difficult to synthesize γ-PGA from seven-membered glutamic acid γ-*N*-carboxy anhydride (NCA), because the compound is so unstable that it easily releases CO_2 to afford pyroglutamate without forming γ-PGA (Sanda et al. 2001). To overcome these problems, Endo et al. synthesized γ-glutamic acid 16-mer α-ethyl ester in stepwise transesterification of the ethyl ester group into benzyl ester group, followed by hydrogenation afforded γ-glutamic acid 16-mer with free carboxyl groups (Sanda et al. 2002). It is expected that chemically synthesized nonracemized γ-PGA would show highly controlled properties and functions.

γ-PGA is water soluble, biodegradable, edible, and nontoxic for humans and the environment. Unlike general poly(amino acid)s, γ-PGA has unique characteristics on enzymatic degradation and immunogenicity. It has been reported that γ-PGA is resistance against many proteases because γ-linked glutamic acids are not easily recognized by common proteases (Oppermann et al. 1998; Obst and Steinbuchel 2004). Moreover, several studies have shown that γ-PGA by itself is a poor immunogen and does not induce booster responses, probably because of its simple homopolymeric structure, similar to those of polysaccharides (Schneerson et al. 2003; Rhie et al. 2003; Wang et al. 2004; Joyce et al. 2006; Kubler-Kielb et al. 2006). Therefore, the potential applications of γ-PGA and its derivatives have been of interest in a broad range of fields, including the medicine, food, cosmetic, and water treatment (Shih and Van 2001). In this review, we summarize the preparation of multifunctional γ-PGA and their pharmaceutical and biomedical applications as drug carriers and tissue-engineering materials.

2 Poly(γ-Glutamic Acid)–Drug Conjugates

Chemotherapy for cancers is usually limited by the toxicity of drugs to normal tissues. The design of polymer–drug conjugates provides a synthetic approach that can overcome some of the problems. Several synthetic polymer-based anticancer drug conjugates have entered clinical studies (Maeda 2001; Li 2002; Li and Wallace 2008). Unlike other synthetic polymers that have been tested in clinical studies, γ-PGA is unique in that it is composed of naturally occurring glutamic acid linked

together through amide bonds rather than a nondegradable C–C backbone. The free carboxyl group in each repeating unit of glutamic acid provides functionality for drug attachment. These features make γ-PGA a promising candidate as a carrier of polymer–drug conjugates for selective delivery of chemotherapeutic agents.

2.1 γ-PGA–Anticancer Drug Conjugates

In the mid-1970s, Ringsdorf propounded a polymer–drug conjugate model that could enhance the delivery of an anticancer drug to a tumor (Ringsdorf 1975). In this model, it was predicted that when an anticancer drug is conjugated to a polymeric carrier, its pharmacological properties could be manipulated by changing the physicochemical properties of the polymer. For example, an insoluble drug can be made water-soluble by introducing solubilizing moieties into the polymer. Likewise, active targeting is possible if a targeting moiety is introduced into the polymer (Fig. 1). To date, several synthetic polymers conjugated with anticancer drug have been successfully introduced into clinical practice, including polystyrene– maleic anhydride copolymer (SMA) (Maeda et al. 2001), *N*-(2-hydroxypropyl)-methacrylamide copolymer (HPMA) (Minko et al. 2000), and poly(α-L-glutamic acid) (α-PGA) (Li et al. 1998) (Fig. 2). A related, but structurally different, α-PGA comprised of glutamic acid is γ-PGA. In this polymer, L-glutamic acid monomers are linked via amide bonds between γ-carboxyl and α-amino groups of adjacent monomers. The polymer is not degraded by various proteases, but is cleaved to glutamic acid monomers under mild acid, where α-PGA remains mostly intact (Weber 1990). Therefore, it is expected that γ-PGA has a unique function as polymer–drug conjugates.

Kishida et al. prepared a conjugate of γ-PGA (1,660 kDa) with 5-fluorouracil (5-FU) [γ-poly(α-hydroxymethyl-5-fluorouracil-glutamate)] (γ-PFUG) as a polymeric prodrug matrix (Fig. 3). 5-FU is a chemotherapeutic drug that is used widely for the treatment of malignant cancers, and is usually the first choice of drug for the treatment of hepatic cellular cancer. But its use has been limited by its systemic toxicities, which have severe gastrointestinal toxicities, hematologic side effects,

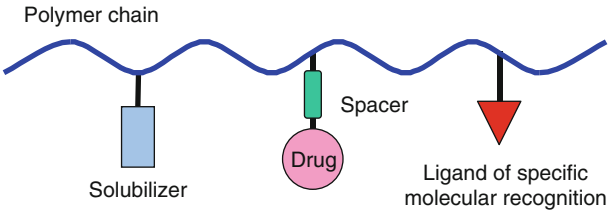


Fig. 1 Basic concept of polymeric drug (Ringsdorf's model). Model for targetable polymer–drug conjugates according to Ringsdorf, in which a solubility enhancer, a pharmacokinetic modifier, a homing device, and specific drugs could be attached to the same polymeric chain

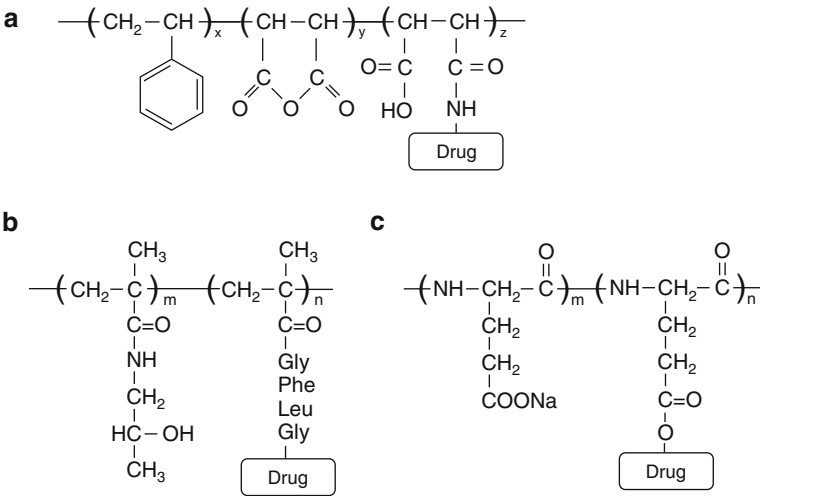


Fig. 2 Polymer–drug conjugates. Chemical structures of drug conjugated (**a**) polystyrene-maleic anhydride copolymer (SMA), (**b**) *N*-(2-hydroxypropyl) methacrylamide copolymer (HPMA), and (**c**) poly(α-L-glutamic acid) (α-PGA)

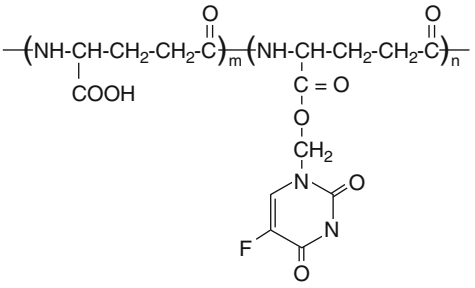


Fig. 3 Chemical structure of γ-PGA-5-FU [γ-poly (α-hydroxymethyl-5-fluorouracil-glutamate)] (γ-PFUG) conjugate

and severe disturbance in bone marrow (Di Paolo et al. 2001). Moreover, 5-FU has a serum half-life of only 15 min, further limiting its usefulness (Jin et al. 2005). In order to maximize the therapeutic effect of 5-FU and minimize its adverse effects, γ-PFUG that can achieve controlled and sustained release and targeting of 5-FU were made. A mixture of 1-, 3-, and 1,3-dihydroxymethyl-5-fluorouracil was introduced to carboxyl group of γ-PGA via ester bond. γ-PFUG films were prepared by casting from DMF. The γ-PFUG matrix degraded in 7 days and incorporated 5-FU was released in 3 days. 5-FU release was improved by mixing free 5-FU into γ-PFUG (Kishida et al. 1998a). Moreover, a slow release of 5-FU was achieved using a γ-PGA benzyl ester (γ-PBG) prepared by esterification of γ-PGA and benzyl bromide as the matrix, which is more hydrophobic in nature. By selecting adequate amount of γ-PFUG or γ-PBG, one can control the 5-FU release *in vitro* from a range in a few hours to over 20 days (Kishida et al. 1998b). γ-PGA conjugated

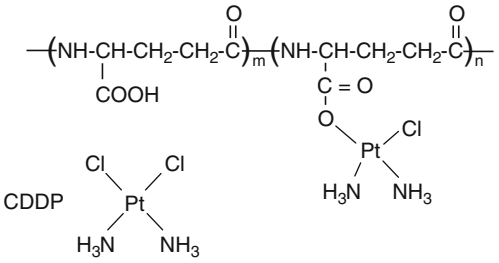


Fig. 4 Chemical structure of γ-PGA–cisplatin (CDDP: cis-dichlorodiamineplatinum (II)) conjugate

with a drug is useful as a biodegradable polymeric prodrug, and may be the basis for a oral drug delivery system (DDS).

Cisplatin (*cis*-dichlorodiamineplatinum(II), CDDP) is one of the extensively used chemotherapeutic agents for the treatment of different cancers such as testicular cancer, ovarian cancer, bladder cancer, lymphoma, and glioma. However, severe side effects including acute nephrotoxicity and chronic neurotoxicity have limited the clinical use of CDDP (Pinzani et al. 1994). To reduce these side effects and enhance the anticancer activity, γ-PGA–CDDP conjugate was prepared (Ye et al. 2006). CDDP was chemically conjugated to γ-PGA (45–60 kDa) through the displacement of CDDP chlorine atoms by hydrogen of carboxyl groups on γ-PGA (Fig. 4). CDDP could be easily conjugated into γ-PGA through a covalent bond. The yield of CDDP incorporation into the γ-PGA was 12.3%. The CDDP was released in the initial 6 h in a burst manner, and thereafter in a sustained manner. Accompanied by the CDDP release, γ-PGA itself was dissociated into monomers, allowing excretion from the body. The γ-PGA–CDDP not only reduced the toxicity of the CDDP but also produced desirable pharmacokinetics and enhanced antitumor activity. α-PGA has been previously used as a carrier to conjugate camptothecin (CPT) against resistant human lung cancer; this previous preparation involved several chemical steps with a chemical component as the initial synthetic material (Zou et al. 2001). γ-PGA–CDDP has advantage that γ-PGA can be obtained in large quantities with high quality and high purity by biosynthesis methods without any chemical modification steps. The PGA–CDDP conjugate was a promising compound for avoiding the toxicity of platinous drugs, while retaining potent antitumor activity.

2.2 γ-PGA–Antidiabetic Drug (Phloridzin) Conjugates

Phloridzin, which is found in the bark and stems of apple trees, is known to inhibit glucose transport competitively through the binding of the glucose moiety to the Na^+/glucose cotransporter (SGLT). Therefore, phloridzin is expected to be used for antidiabetic drug. However, phloridzin has not been used as an oral antidiabetic drug because toxic phloretin is released through the hydrolysis of a glucoside bond

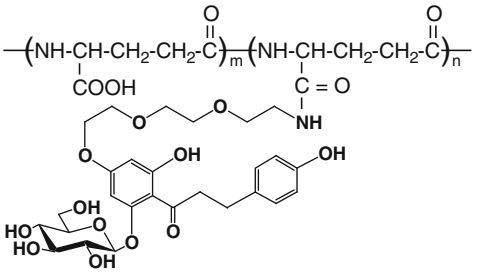


Fig. 5 Chemical structure of γ-PGA–phloridzin (PGA–PRZ) conjugate. Phloridzin was introduced via a ω-amino triethylene glycol linker to γ-PGA

of phloridzin by intestinal β-glucosidase (Tsujihara et al. 1996). This hydrolysis also results in a reduction of phloridzin activity because of the lack of intramolecular glucose residues. Although low-molecular weight-phloridzin analogs and phloretin derivatives have been examined, promising results have not been obtained yet (Asano et al. 2004). To overcome this problem, Sakuma et al. designed and prepared a novel γ-PGA (58 kDa)–phloridzin conjugate, via a ω-amino triethylene glycol linker (Fig. 5) (Ikumi et al. 2008). The potential of γ-PGA–phloridzin conjugate (PGA–PRZ) obtained as a novel oral antidiabetic drug was examined by *in vitro* and *in vivo* experiments. A PGA–PRZ with a 15% phloridzin content inhibited glucose transport from mucosal to serosal sides of the everted rat's small intestine, and its inhibitory effect was as strong as that of intact phloridzin. When the PGA–PRZ was given orally to rats before glucose administration, the glucose-induced hyperglycemic effect was significantly suppressed. On the other hand, reduction of an increase in the blood glucose concentration was scarcely observed when the PGA–PRZ was substituted with a double amount of intact phloridzin. This difference in the biological activity between PGA–PRZ and intact phloridzin resulted from the improved stability of a glucoside bond of phloridzin through the conjugation with γ-PGA (Sakuma et al. 2009). D-glucose, which is essential for the inhibition of SGLT1, was not released from PGA–PRZ with a phloridzin content of greater than 15%, even though an immediate release of D-glucose from intact phloridzin was observed, and the toxic phloretin was not released from the conjugate. These results suggest that the γ-PGA–phloridzin conjugate has potential as oral antidiabetic drugs with high safety.

2.3 γ-PGA–F(ab′) Antibody Conjugates

Several systems have been developed to restrict the delivery of the chemotherapeutic agent to the tumor site. With the identification of cell-specific receptor/antigens on tumor cells and tumor endothelial cells, it has been possible to actively target chemotherapeutic or antiangiogenic agents using ligand- or antibody-bearing delivery systems (Satchi-Fainaro et al. 2000). Targeting of cytotoxic drugs via comodification of the drug to a water-soluble polymer with a cell-specific antibody is an attractive

approach for anticancer chemotherapy. Tumor-specific antibody F(ab′) fragments have been successfully conjugated to HPMA with cytotoxic agents doxorubicin or adriamycin to give cytotoxic conjugates (Jelinkova et al. 1998; Kunath et al. 2000).

Prodhomme et al. have established the feasibility of attaching bioactive molecules and macromolecules such as cell-specific antibody F(ab′) fragments to γ-PGA (Prodhomme et al. 2003). Using synthetic linkers, the α-carboxylate side chains of γ-PGA were conjugated to an exposed thiol side chain of an antibody F(ab′) fragment, a monoclonal rat IgG2a antibody (mAb) with activity against idiotypic determinants carried by the IgM molecule of the BCL1 lymphoma. The most successful linker was found to be maleimide linker, containing an ethylene glycol spacer capable of hydrogen bonding with the aqueous solvent. The γ-PGA–antibody conjugate could retain its biological recognition properties, but showed reduced binding efficacy and cell growth inhibition, compared with the free antibody. γ-PGA was also conjugated with the free amino group of glycopeptide antibiotic vancomycin. The γ-PGA–vancomycin conjugate showed slightly lower antibacterial activity than free vancomycin. In this case, however, there is some indication of enhanced biological activity against vancomycin-resistant strains, against which the free ligand shows low affinity. The observation that the immobilized ligands retain their biological activity suggests that γ-PGA–antibody conjugates could in principle be used to target cytotoxic drugs in the human body. γ-PGA provide interesting alternatives to synthesis polymers for antibody F(ab′) immobilization, which may prove to have certain advantages for biocompatibility.

3 Self-Assembled Nanoparticles for Drug Delivery System

Nanoparticles prepared from synthetic or natural polymers have applications in various technological and biomedical fields, because their chemical structures, surface functionalities, and particle size can be easily controlled. Nanoparticles with entrapped therapeutic agents, such as small molecules, peptides, proteins, and DNA, have recently been shown to possess significant potential as DDS (Hans and Lowman 2002; Allen and Cullis 2004; Torchilin 2006; Vasir and Labhasetwar 2007; Mundargi et al. 2008). Particulate delivery systems are concerned with the systematic release of a pharmaceutical agent to maintain a therapeutic level of the drug in the body for a sustained period of time. This may be achieved by incorporating the therapeutic agent into a degradable polymer particle, releasing the agent continuously as the matrix degradation (Edlund and Albertsson 2000). DDS with nanoparticles is one of the most promising because it may reduce unwanted toxic side effects and improve the therapeutic effect.

Self-assembling polymer or block/graft copolymers that can form nanostructure have been extensively investigated in the field of biotechnology and pharmaceuticals. In general, hydrophobic interactions, electrostatic forces, hydrogen bonds, van der Waal forces, or combinations of these interactions are available as the driving forces for the formation of the polymer complexes (Kakizawa and Kataoka

2002; Zhang and Eisenberg 1995; Dou et al. 2003; Reihs et al. 2004; Kang et al. 2005). Numerous investigators have shown that the biological distribution of drugs, proteins, and DNA can be modified, both at the cellular and organ levels, using micro/nanoparticles delivery systems. Recently, many studies have focused on self-assembled biodegradable nanoparticles for biomedical and pharmaceutical applications. In particular, poly(amino acid)s have received considerable attention for their medical applications as potential polymeric drug carriers. Several amphiphilic block and graft copolymers based on poly(amino acid) have been employed such as poly(L-glutamic acid) (Holowka et al. 2005), poly(L-aspartic acid) (Arimura et al. 2005), poly(L-lysine) (Akiyoshi et al. 2000), poly(L-arginine) (Holowka et al. 2007), and poly(L-asparagine) (Jeong et al. 2003) as hydrophilic segments, and poly(β-benzyl-L-aspartate) (Kataoka et al. 2000), poly(γ-benzyl-L-glutamate) (Lin et al. 2007a), and poly(L-histidine) (Lee et al. 2003) as hydrophobic segments. Amphiphilic copolymers based on poly(amino acid) form micelles through self-association in water. γ-PGA or its derivatives have also been found to form self-assembled, nano-sized particles, and are applied for biomedical fields.

3.1 γ-PGA-Graft–Phenylalanine Copolymer

Nanoparticles fabricated by the self-assembly of amphiphilic block copolymers or hydrophobically modified polymers have been explored as drug carrier systems (Akagi et al. 2007a; Wang et al. 2007a).

In general, these amphiphilic copolymers consisting of hydrophilic and hydrophobic segments are capable of forming polymeric structures in aqueous solutions via hydrophobic interactions. These self-assembled nanoparticles are composed of an inner core of hydrophobic moieties and an outer shell of hydrophilic groups (Gaucher et al. 2005; Letchford and Burt 2007).

Akashi et al. prepared nanoparticles composed of hydrophobically modified poly (γ-glutamic acid) (γ-PGA) (Matsusaki et al. 2004; Kaneko et al. 2005). γ-PGA as the hydrophilic backbone and L-phenylalanine (Phe) as the hydrophobic segment were synthesized by grafting Phe to γ-PGA using water-soluble carbodiimide (WSC) (Fig. 6). The γ-PGA-*graft*–Phe copolymer (γ-PGA–Phe) with more than 50% grafting degree formed monodispersed nanoparticles in water due to their amphiphilic properties. To prepare nanoparticles, γ-PGA–Phe dissolved in DMSO was added to various concentration of NaCl solution, and then the resulting solutions were dialyzed and freeze-dried. The γ-PGA–Phe formed monodispersed nanoparticles, and the particle size of the γ-PGA–Phe nanoparticles could be easily controlled (30–200 nm) by changing NaCl concentration (Kim et al. 2009) (Fig. 7). The nanoparticles showed a highly negative zeta potential (−25 mV) due to the ionization of the carboxyl groups of γ-PGA located near the surfaces. The specific self-assembly behavior of γ-PGA–Phe in aqueous solution was due to multiple phenyl groups stacking. Beside the particle formation of γ-PGA by using hydrophobic interaction, nanoparticles formed by complexation of γ-PGA with bivalent

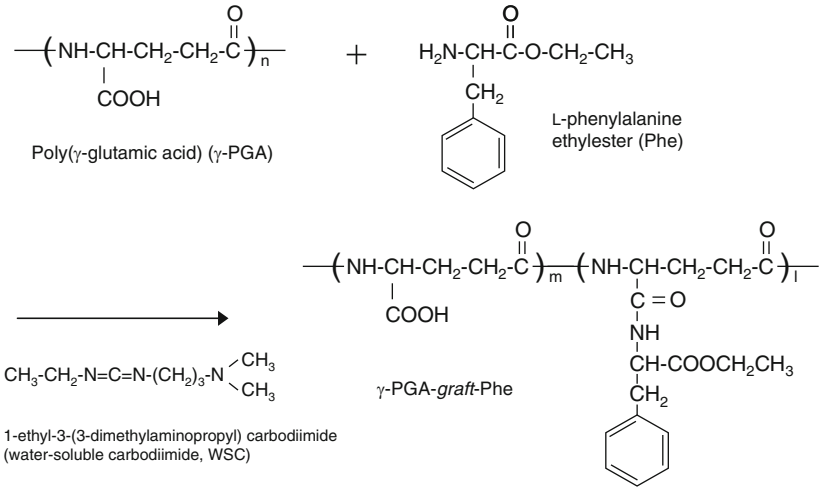


Fig. 6 Synthesis of γ-PGA-*graft*-Phe (γ-PGA–Phe). γ-PGA was hydrophobically modified by Phe in the presence of WSC

metal ion complex (Bodnar et al. 2008) or chemical cross-linking of carboxyl group of γ-PGA (Radu et al. 2008) have been reported.

The hydrolytic and enzymatic degradation of the γ-PGA–Phe nanoparticles *in vitro* was studied by gel permeation chromatography (GPC), scanning electron microscopy (SEM), dynamic light scattering (DLS), and ^{1}H-NMR measurements (Akagi et al. 2005a, 2006a). The hydrolysis ratio of γ-PGA and these hydrophobic derivatives was found to decrease upon increasing the hydrophobicity of the γ-PGA derivates. The degradation of the γ-PGA backbone by γ-glutamyl transpeptidase (γ-GTP) resulted in a dramatic change in nanoparticle morphology. With increasing time, the γ-PGA–Phe nanoparticles began to decrease in size and finally disappeared completely. The enzymatic degradation (pronase E, protease, cathepsin B, and lipase) of the nanoparticles occurred via the hydrolysis of γ-PGA as the main chain, and Phe as the side chain. Sutherland et al. reported that γ-PGA was degraded in macrophages starting 4 h after uptake with continued degradation occurring for at least 24 h (Sutherland et al. 2008; Sutherland and Kozel 2009). Nanoparticles composed of biodegradable amphiphilic γ-PGA with reactive function groups can undergo further modification, and are expected to have a variety of potential pharmaceutical and biomedical applications, such as drug or vaccine carriers.

The capture of water-soluble drugs such as proteins in the nanoparticle carrier system can be carried out through various approaches. Double-emulsion solvent evaporation/extraction is a common and convenient method for the encapsulation of proteins into a polymer matrix (Quellec et al. 1998). However, the possible denaturation of the proteins at the oil–water interface limits the usage of this method. It has been reported that this interface causes conformational changes in bovine serum albumin (BSA) (Sah 1999; Panyam et al. 2003). The prevention of

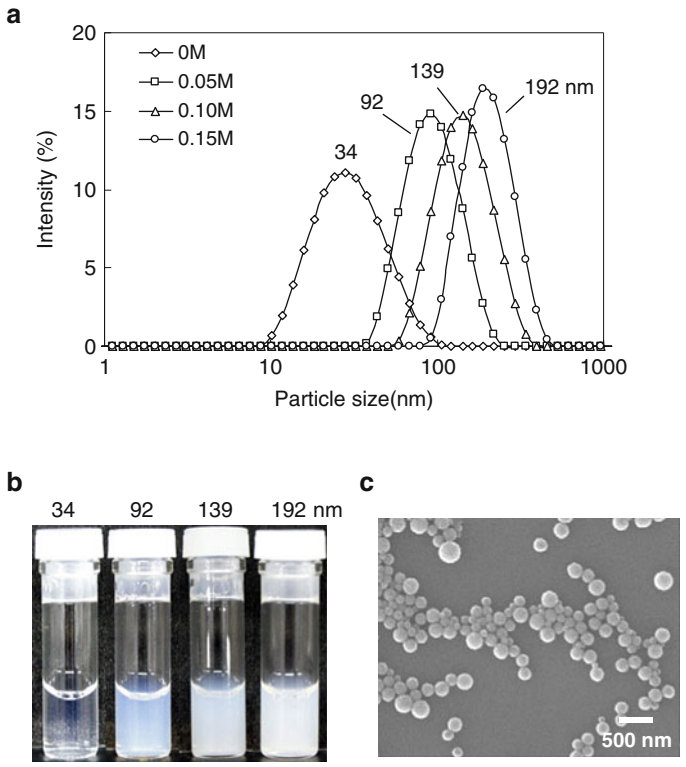


Fig. 7 (**a**) Size changes of γ-PGA–Phe nanoparticles prepared at various NaCl concentrations. The size of nanoparticles was measured by dynamic light scattering (DLS). (**b**) Photographs of γ-PGA–Phe nanoparticles (2.5 mg/ml) dispersed in water. (**c**) Scanning electron microscope (SEM) image of γ-PGA–Phe nanoparticles prepared by 0.15 M NaCl solution

protein denaturation and degradation, as well as high entrapment efficiency, would be of particular importance in the preparation of nanoparticles containing water-soluble drugs such as a protein. Therefore, this novel type of nanoparticle needs to be developed.

Protein-loaded γ-PGA–Phe nanoparticles were prepared by encapsulation, covalent immobilization, or physical adsorption methods (Fig. 8) to study their potential applications as protein carriers (Akagi et al. 2005b, 2006b). To prepare the protein-encapsulated γ-Phe–Phe nanoparticles, 0.25–4 mg of protein [thyroglobulin, catalase, concanavalin A (Con A), bovine serum albumin (BSA), ovalbumin (OVA), peroxidase, β-lactoglobulin, myoglobin, lysozyme, α-lactalbumin, and cytochrome c] was dissolved in 1 ml of saline, and 1 ml of the γ-PGA–Phe (10 mg/ml in DMSO) was added to the protein solutions. The resulting solutions were centrifuged and repeatedly rinsed. The encapsulation of proteins of various molecular weights and isoelectric points into the nanoparticles was successfully achieved. All proteins used in this experiment were successfully encapsulated into the nanoparticles. The

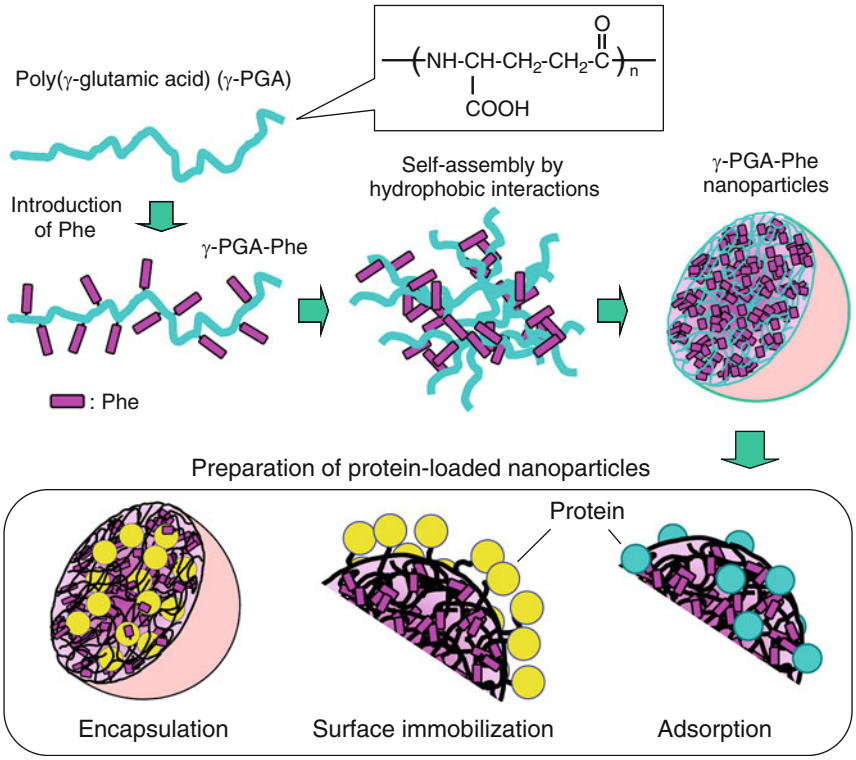


Fig. 8 Preparation of protein-encapsulated, -immobilized, or -adsorbed γ-PGA–Phe nanoparticles

encapsulation efficiency was found to be in the range of 30–60% for most samples. For all samples tested, it was observed that the encapsulation efficiency for a given protein was not markedly influenced by the physical properties of that protein. The size of the nanoparticles was increased when various proteins were encapsulated. When OVA was encapsulated into the nanoparticles (90 μg/mg NP), the size of nanoparticle increased from 180 to 256 nm. These results might be due to an increase in the swelling capacity of the nanoparticles due to the hydrophilic properties of the protein. The OVA encapsulated into the nanoparticles was not released (less than 10%) over the pH range of 4–8, even after 10 days. Moreover, it was found that the γ-PGA–Phe nanoparticles have some excellent properties, as follows. The enzyme-encapsulated nanoparticles showed high enzymatic activity. In the case of protein-encapsulated nanoparticles prepared by the self-assembly of γ-PGA–Phe, the encapsulated protein may be more stable than via the emulsion method. Proteins encapsulated into the nanoparticles appear to be adequate in terms of the preservation of the protein structure. The γ-PGA–Phe nanoparticles and protein-encapsulated nanoparticles could be preserved by freeze-drying process. The results of cytotoxicity tests showed that the nanoparticles did not cause any

relevant cell damage. Therefore, it is expected that the γ-PGA–Phe nanoparticle will have great potential as multifunctional carriers in pharmaceutical and biomedical applications, such as drug and vaccine delivery systems. Also, Portilla-Arias et al. reported the preparation of nanoparticles made of alkyl esters of γ-PGA and their potential application as drug and protein carrier (Portilla-Arias et al. 2009).

Polymeric nanoparticles with entrapped antigens represent an exciting approach to control the release of vaccine antigens and to optimize the desired immune response via selective targeting of the antigen to antigen-presenting cells (APCs) (Panyam and Labhasetwar 2003; Jilek et al. 2007). The submicron size-range of these delivery systems offers a number of distinct advantages over microparticles, including relatively higher intracellular uptake compared with microparticles (Harding and Song 1994; Nixon et al. 1996). Dendritic cells (DCs) are considered to be initiators and modulators of immune responses and are capable of processing antigens through both major histocompatibility complex (MHC) class I and II pathways (Banchereau and Steinman 1998). Therefore, the delivery of therapeutic macromolecules, such as proteins, peptides, and DNA, to DCs and the activation of the antigen-presenting pathway are some of the most important issues in the development of effective vaccines (Gamvrellis et al. 2004). Moreover, adjuvants that possess the ability to induce of DC maturation are useful for DC-based immunotherapy (Elamanchili et al. 2004).

Akagi et al. demonstrated the use of nanoparticles composed of amphiphilic poly (amino acid) derivatives as vaccine delivery and adjuvants (Akagi et al. 2007b; Uto et al. 2007, 2009a). To evaluate the uptake of OVA-encapsulated γ-PGA–Phe nanoparticles (OVA-NPs) by DCs, murine bone marrow-derived DCs were incubated with 250-nm-sized OVA-NPs for 30 min at 37°C. The cells were then analyzed by flow cytometry (FCM). OVA-NPs were efficiently taken up into DCs, whereas the uptake of OVA alone was barely detectable at the same concentration of OVA (Fig. 9). OVA-NPs were more efficiently taken up than OVA alone by DCs,

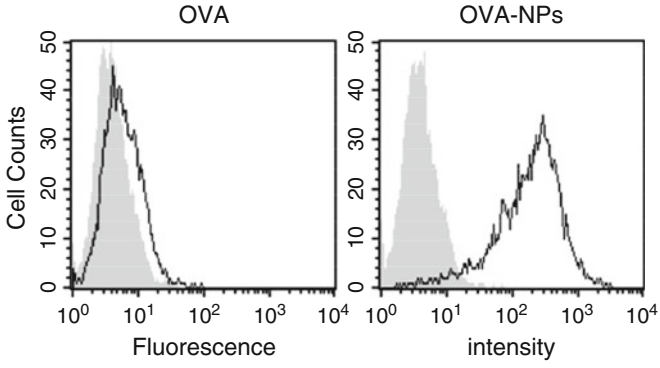


Fig. 9 Uptake of OVA-encapsulated γ-PGA–Phe nanoparticles (OVA-NPs) by dendritic cells (DCs). Mouse bone marrow-derived DCs were incubated in the absence (*gray histogram*) or presence (*line histogram*) of 250-nm-sized OVA-NPs or OVA alone (25 μg/ml) for 30 min at 37°C. The cells were then analyzed by flow cytometry (FCM)

and the uptake of OVA-NPs was inhibited at 4°C. These results suggest that OVA-NPs were phagocytosed mainly via endocytosis by the DCs. In the case of OVA alone, an approximately 30-fold higher concentration was required to elicit a similar amount of intracellular OVA as compared with OVA-NPs.

The maturation of DCs is associated with increased expression of several cell surface markers, including the costimulatory molecules CD40, CD80, CD83, CD86, MHC class I, and class II (Banchereau and Steinman 1998). The maturation of DCs is deeply involved in their ability to process and present antigens to T cells. To determine whether the uptake of γ-PGA–Phe nanoparticles mediates the phenotypic maturation of DCs, DCs were incubated with γ-PGA–Phe nanoparticles for 48 h, and the expression of surface molecules was measured by FCM. Upon exposure of these DCs to the nanoparticles, the expression of CD86 and MHC class I was increased in a dose-dependent manner. The expression levels of costimulatory molecules in NPs-pulsed DCs were similar to those of lipopolysaccharide (LPS)-pulsed DCs. These results suggest that γ-PGA–Phe nanoparticles have great potential as adjuvant for DC maturation. The mechanisms responsible for DC maturation by γ-PGA–Phe nanoparticles are still unclear. However, it is hypothesized that not only the uptake process of nanoparticles but also the characteristics of polymers consisting of nanoparticles are important for the induction of DC maturation. In addition, soluble γ-PGA-induced innate immune responses in Toll-like receptor 4 (TLR4)-dependent manner in DCs have been reported (Kim et al. 2007; Lee et al. 2009). Treatment with high molecular weight γ-PGA induced a significant upregulation of CD40, CD80, and CD86 expression in wild-type DCs. In contrast, DCs from TLR4-defective mice did not show an enhanced expression of maturation makers in response to the 2,000 kDa γ-PGA treatment. It is suggested that the γ-PGA–Phe nanoparticles also induce DC maturation in a TLR4-dependent manner using the same 2,000 kDa γ-PGA, because γ-PGA is located near the nanoparticle surfaces.

Induction and regulation of an adaptive immune response by vaccination is possible for a broad range of infectious diseases or cancers. Vaccine delivery or adjuvant that can induce antigen-specific humoral and cellular immunity is useful for development of effective vaccine systems. Cellular immunity is required to remove intracellular pathogens, while humoral immunity plays a central role in neutralizing extracellular microorganisms. The efficacy of antigen (Ag)-encapsulated γ-PGA–Phe nanoparticles on the induction of Ag-specific cellular and humoral immune responses was examined using OVA as a model Ag (Uto et al. 2007, 2009b). The immune responses were investigated in mice after subcutaneous immunization with OVA-encapsulated γ-PGA–Phe nanoparticles (OVA-NPs). Ag-specific cytotoxic T lymphocyte (CTL) response was not observed in the spleen cells obtained from the control (PBS) and OVA-immunized mice. In contrast, the spleen cells obtained from the mice immunized with OVA-NPs showed a more potent Ag-specific CTL response than those obtained from mice immunized with OVA plus complete Freund's adjuvant (CFA). When Ag-specific antibody responses were examined and compared among the groups after immunization, both OVA-NP- and OVA mixed with CFA (OVA + CFA)-immunized mice showed significantly higher levels of OVA-specific total IgG, IgG1, and IgG2a

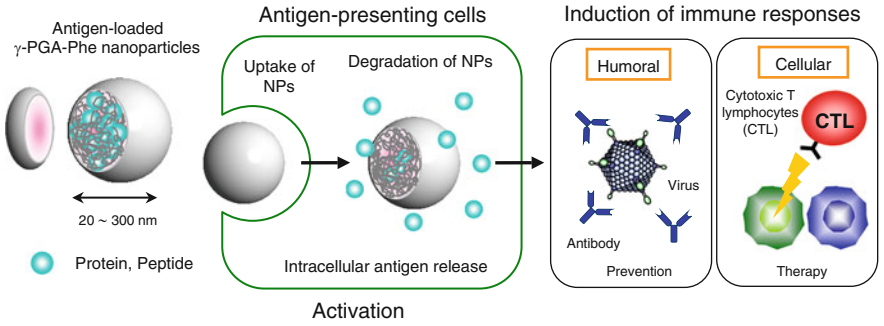


Fig. 10 Induction of immune responses by nanoparticle-based vaccine

antibodies than OVA-immunized mice. These results indicate that the γ-PGA–Phe nanoparticles have the ability to prime cellular and humoral immunity by vaccination. It has been demonstrated that the γ-PGA–Phe nanoparticles are also effective for vaccines against human immunodeficiency virus (HIV) (Wang et al. 2007b, 2008), influenza virus (Okamoto et al. 2007, 2009), Japanese encephalitis virus (Okamoto et al. 2008), human T-cell leukemia virus Type-I (HTLV-I) (Matsuo et al. 2007), or cancers (Yoshikawa et al. 2008a, b). The antigen-loaded γ-PGA–Phe nanoparticles would provide an efficient antigen delivery and adjuvant system for vaccination against viral infections or tumors (Fig. 10).

3.2 γ-PGA–Poly(lactide) Block Copolymer

The amphiphilic self-assembled nanoparticles are composed of a hydrophobic inner core and a hydrophilic outer shell. Core-forming hydrophobic block may affect the drug loading capacity and its release kinetics as a result of hydrophobic interactions between drugs and polymers. In contrast, the hydrophilic shell-forming block determines surface properties of the nanoparticles and influences interactions between the surrounding environments and the nanoparticles (Nam et al. 2003). Biodegradable polymer, such as poly(lactide) (PLA), poly(ε-caprolactone) (PCL), poly(β-benzyl L-aspartate) (PLBA), and poly(γ-benzyl L-glutamate) (PLBG), has been used mostly for the core-forming hydrophobic segment of nanoparticles. On the other hand, poly(ethylene oxide) (PEO), a nontoxic and highly hydrated polymer, has been used as the outer shell segment of nanoparticles because of its superior biocompatibility (Lee et al. 2004).

Sung et al. synthesized amphiphilic block copolymers composed of γ-PGA as the hydrophilic segment and PLA as the hydrophobic segment to prepare a novel type of self-assembled nanoparticles. Block copolymers composed of γ-PGA (4 kDa) and PLA (10 kDa) (γ-PGA–PLA) were synthesized using *N*,*N*-carbonyldiimidazole (CDI) to activate the terminal hydroxyl group of PLA. γ-PGA was successfully conjugated to PLA by CDI (Fig. 11) (Liang et al. 2005). γ-PGA–PLA

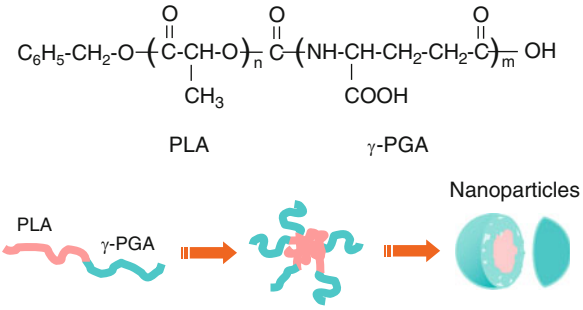


Fig. 11 Chemical structure of γ-PGA–PLA block copolymer and the formation of self-assembled γ-PGA–PLA nanoparticles

nanoparticles were prepared using the emulsion/solvent evaporation technique. The block copolymers (10 mg) were dissolved in 1 ml methylene chloride, then vortexed and emulsified in 10 ml of a 0.1 wt.% sodium cholate solution using a sonicator (cycles of 1 s sonication followed by 1 s of pauses, total time 5 min). Afterward, the solvent was evaporated in a vacuum oven at 37°C for 1 h. The nanoparticles were then recovered by centrifugation for 20 min at 20,000 × g at 4°C. Subsequently, the nanoparticles were resuspended by PBS (pH 7.4). The prepared nanoparticles had a mean particle size of about 140 nm, and negative surface charge with a zeta potential of about −20 mV due to the carboxyl groups on the hydrophilic γ-PGA shell. In the stability study, no aggregation or precipitation of nanoparticles was observed during storage for up to 1 month, as a result of the electrostatic repulsion between the negatively charged nanoparticles. For the potential of targeting liver cancer cells, the prepared γ-PGA–PLA nanoparticles were further conjugated with galactosamine. Galactosamine was conjugated to the surface of nanoparticles via an amide linkage by *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) in the presence of *N*-hydroxysuccinimide (NHS). Hepatocytes are known to recognize galactose and *N*-acetylgalactosamine-terminated glycoproteins via the asialoglycoprotein (ASGP) receptors located on their surfaces. It was reported that the ASGP receptors are also abundantly expressed on the surfaces of various hepatoma cell lines (Fallon and Schwartz 1989). To assess the extents of internalization of the prepared galactosamine-immobilized γ-PGA–PLA nanoparticles (Gal-NPs) into HepG2 cells (a liver cancer cell line), the Gal-NPs were labeled with rhodamine-123. With increasing the galactosamine content conjugated on the nanoparticles, the uptake of Gal-NPs by HepG2 cells increased significantly. In contrast, the Gal-NPs were not taken up by Hs68 cells (without ASGP receptors). These results indicated that the galactosylated nanoparticles had a specific interaction with HepG2 cells via ligand–receptor recognition. The γ-PGA–PLA nanoparticles conjugated with galactosamine may be a potential DDS for targeted delivery to liver cancers.

Paclitaxel (Taxol) is one of the most active anticancer drugs used in cancer chemotherapy. However, the use of paclitaxel is limited by its toxicity and limited

aqueous solubility. Paclitaxel is highly hydrophobic and has a solubility of approximately 1 μg/ml in aqueous solution at pH 7.4. It is currently formulated as a concentrated solution paclitaxel/Cremophor EL/ethyl alcohol, but several toxic effects have been attributed to Cremophor EL, including serious hypersensitivity reactions. Therefore, numerous attempts have been made to develop drug formulations with reduced systemic toxicity and an enhanced therapeutic index by using water or other vehicles (Li et al. 2000). Paclitaxel-loaded γ-PGA–PLA nanoparticles were prepared to study the potential of targeting liver cancer cells (Liang et al. 2006a). The paclitaxel-loaded nanoparticles with various feed weight ratios of paclitaxel to block copolymer (P/BC ratio) were produced using an emulsion/solvent evaporation technique. Increasing the P/BC ratio significantly increased the drug loading content of the prepared nanoparticles. In the case of P/BC ratio of 1/10, the particle size, zeta potential, drug loading content, and loading efficiency was 129 nm, −20 mV, 5%, and 54%, respectively, and showed efficiently loading efficiency. Moreover, the size of nanoparticles increased with increasing loading content of drug. When the P/BC ratio was increased from 0/10 to 3/10, the size of nanoparticles increased from 115 to 263 nm. The loaded paclitaxel was continuously released from nanoparticles prepared with distinct P/BC ratios. All samples released a burst of paclitaxel in the initial stage. An amount of 10–25% of the loaded drug was released in the first hour. This may be due to some of the drugs that were deposited near the γ-PGA shell of the prepared nanoparticles. As the P/BC ratio increased, the rate of release of paclitaxel from the prepared nanoparticles decreased significantly. It was reported that a hydrophobic drug encapsulated within nanoparticles partially crystallized at a higher drug loading content, while it formed a molecular dispersion at a lower drug loading content (Gref et al. 1994). For the potential of targeting liver cancer cells, galactosamine was further conjugated on the prepared paclitaxel-loaded γ-PGA–PLA nanoparticles (Gal–P-NPs) as a targeting moiety. It was found that the activity in inhibiting the growth of HepG2 cells (a liver cancer cell line) by the Gal–P-NPs was comparable to that of a clinically available paclitaxel formulation (Phyxol). These results suggested that the Gal–P-NPs are internalized into HepG2 cells via receptor-mediated endocytosis, inhibiting the growth of cells. Moreover, the biodistribution of the prepared Gal–P-NPs was studied *in vivo* in normal mice and hepatoma-tumor-bearing nude mice (Liang et al. 2006b). The antitumor efficacy of the prepared nanoparticles in hepatoma-tumor-bearing nude mice was also examined. It was found that the groups injected with Phyxols, the paclitaxel-loaded nanoparticles or the Gal–P-NPs significantly delayed the tumor growth as compared with the nontreated group. Among all studied groups, the group injected with the Gal–P-NPs appeared to have the most significant efficacy in the reduction of the size of the tumor. This is because a large number of the Gal–P-NPs were observed at the tumor site, and subsequently released their encapsulated paclitaxel to inhibit the growth of the tumor. These results indicated that the Gal–P-NPs had a specific interaction with the hepatoma tumor induced in nude mice *via* ligand–receptor recognition. Therefore, the active targeting nature of the prepared Gal–P-NPs is considered to be used as a potential DDS for the targeted delivery to liver cancers.

3.3 γ-PGA–Chitosan Complex

Polymer complexes associated with two or more complementary polymers are widely used in potential applications in the form of particles, hydrogels, films, and membranes. In particular, a polyion complex (PIC) can be easily formed when oppositely charged polyelectrolytes are mixed in aqueous solution and interact via electrostatic (coulombic) interactions (Fig. 12). Nanoscaled structural materials (e.g., nanoparticles, micelles, nanogels, and hollow nanospheres) composed of PIC are prepared by tuning the preparation conditions, such as the charge ratio of the anionic-to-cationic polymers, temperature, concentration, and type of polyelectrolyte (Kakizawa and Kataoka 2002; Muller et al. 2005; Hartig et al. 2007). PIC containing γ-PGA and chitosan (CT) as a cationic polymer has been applied for preparation of nanoparticles, hydrogels, and films for biomedical applications. CT is a polysaccharide constituted by *N*-glucosamine and *N*-acetyl-glucosamine units (Fig. 13), in which the number of *N*-glucosamine units exceeds 50%. CT is positively charged and solubilized by protonation of its amino groups when the solution pH is below 6. CT can be degraded into nontoxic products *in vivo*, and thus it has been widely used in various biomedical applications (Hsieh et al. 2005; Kang et al. 2007).

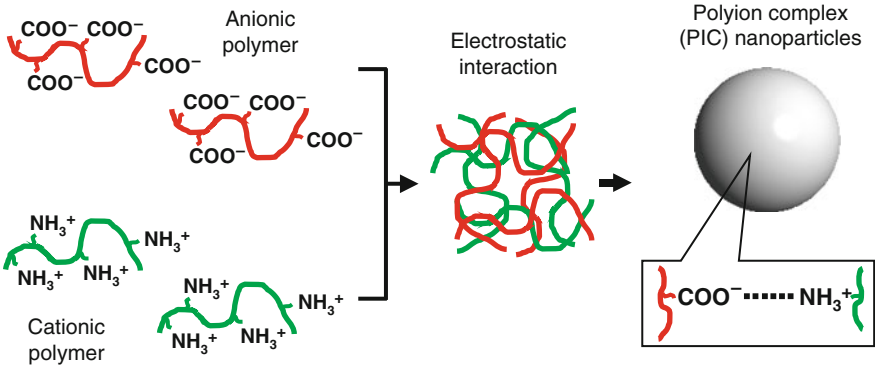
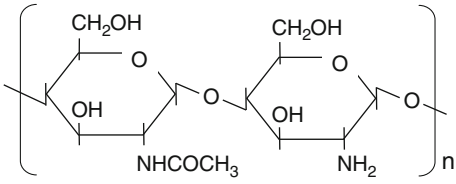


Fig. 12 Formation of polyion complex (PIC) nanoparticles composed of oppositely charged polyelectrolytes *via* electrostatic (coulombic) interactions



Chitosan (β-1, 4-poly-D-gucosamine)

Fig. 13 Chemical structure of chitosan (β-1, 4-poly-D-glucosamine)

Sung et al. investigated the PIC particle formation of γ-PGA and CT by self-assembly in aqueous media (Lin et al. 2005). Nanoparticles were obtained upon addition of a γ-PGA (160 kDa) aqueous solution (pH 7.4) into a low-molecular weight CT (50 kDa) aqueous solution (pH 6.0) at varying concentrations under magnetic stirring. Nanoparticles were collected by ultracentrifugation. The particle sizes and the zeta potential of γ-PGA and CT (γ-PGA–CT) nanoparticles, prepared at varying concentrations of γ-PGA and CT, were determined. It was found that the particle size and the zeta potential of the prepared nanoparticles were mainly determined by the relative amount of the local concentration of γ-PGA in the added solution to the surrounding concentration of CT. At a fixed concentration of CT, an increase in the γ-PGA concentration allowed γ-PGA molecules interacting with more CT molecules and thus formed a larger size of nanoparticles. When the amount of CT molecules exceeded that of local γ-PGA molecules, some of the excessive CT molecules were located onto the γ-PGA–CT nanoparticle surfaces. Thus, the resulting nanoparticles may display a structure of a neutral PIC core surrounded by a positively charged CT shell ensuring the colloidal stabilization. These results clearly indicated that the size (from 80 to 400 nm) and surface charge (from −35 to +25 mV) of γ-PGA–CT nanoparticles could be easily controlled by changing the mixing ratio of two polymers. Hajdu et al. also prepared γ-PGA (1,200 kDa)–CT (320 kDa) nanoparticles (Hajdu et al. 2009). The size and size distribution of the nanoparticles depend on the concentrations of γ-PGA and CT solutions and their ratio as well as on the pH of the mixture and the order of addition. The particle size was in the range of 20–285 nm measured by transmission electron microscopy (TEM), and the average hydrodynamic diameters were between 150 and 330 nm.

However, the stability and characteristics of prepared PIC are influenced by various factors involving their chemical compositions and their surrounding environment. In particular, for PIC micelles or nanoparticles, the ionic strength and pH of the solution is a key parameter for stability because of the shielding effect of the ionic species on the electrostatic interactions (Jaturanpinyo et al. 2004). Therefore, destabilization of PIC under physiological conditions limits their applications as a drug carrier. In fact, in the case of γ-PGA–CT nanoparticles, at pH 7.4, CT was deprotonated and thus led to the collapse or aggregation of nanoparticles. The prepared nanoparticles remained intact in the pH range of 2.5–6.6. To overcome this problem, the stabilization of multi-ion-crosslinked nanoparticles composed of γ-PGA and CT blended with tripolyphosphate (TPP) and $MgSO_4$. By adding $MgSO_4$ and TPP, the stability of nanoparticles at distinct pH values increased significantly (Lin et al. 2008). The multi-ion-crosslinked nanoparticles had a superior stability over a broader pH range than uncrosslinked nanoparticles.

An Oral route is the most convenient and comfortable means of administering protein/peptide drugs such as insulin, because the pain caused by injection could be avoided, leading to a higher patient compliance. However, it is well known that the bioavailability of peptide and protein drugs after oral administration is very low because of their instability in the gastrointestinal (GI) tract and low permeability through the intestinal mucosa (Sakuma et al. 2001). Nanoparticles have been

studied extensively as carriers for oral drug delivery, whose purpose is to improve the bioavailability of drugs with poor absorption characteristics (Damg et al. 1990; Sakuma et al. 1997). To this end, a pH-responsive particulate delivery system that is stable in the gastrointestinal (GI) tract and can swell significantly or degrade and subsequently release the loaded drug when adhering and infiltrating into the mucus of the intestinal tract is desired. Insulin-loaded PIC nanoparticles composed of γ-PGA and *N*-trimethyl chitosan (TMC) (γ-PGA–TMC) were prepared for oral insulin delivery via the paracellular pathway (Mi et al. 2008). The insulin solution was premixed with an aqueous γ-PGA. The mixed solution was then added into an aqueous TMC under magnetic stirring at room temperature. The loading efficiency and loading content of insulin in γ-PGA–TMC nanoparticles were only 20.8%. To increase the amount of insulin loaded, Mg^{2+} was blended in the premixed aqueous insulin/γ-PGA in the preparation of nanoparticles. In the presence of Mg^{2+}, the insulin loading efficiency (73.8%) in γ-PGA–TMC nanoparticles increased significantly due to cross-linkage between insulin and γ-PGA via divalent metal ions. The release profiles of insulin from the insulin-loaded γ-PGA–TMC nanoparticles were investigated at pH 2.5, 6.4, and 7.4, simulating the pH environments of the fasting stomach, the duodenum, and the bloodstream, respectively. At pH 2.5, the amount of insulin released from the nanoparticles was about 20% within the first hour. At pH 6.4, there was only a minimal amount of insulin released from the nanoparticles. At pH 7.4, the nanoparticles were significantly swelled and a sustained release profile of insulin was observed. The release of insulin from the prepared nanoparticles was mainly influenced by their pH-dependent swelling property.

The γ-PGA–TMC, γ-PGA–CT, and γ-PGA–CT blended with TPP and $MgSO_4$ nanoparticles with a positive surface charge (or shelled with TMC or CT) could transiently open the tight junctions between Caco-2 cells *in vitro* and thus increased the paracellular permeability (Lin et al. 2005, 2007b, 2008; Mi et al. 2008). It has been suggested that interactions of the positively charged amino groups of CT with the negatively charged sites on cell surfaces and tight junctions induce a redistribution of F-actin and the tight junction's protein. Moreover, the *in vivo* results clearly indicated that the insulin-loaded nanoparticles could effectively reduce the blood glucose level in a diabetic rat model (Sonaje et al. 2009). The results suggest that γ-PGA–CT nanoparticles may be a suitable carrier for transmucosal delivery of insulin within the entire intestinal tract.

Gene delivery has great potential for the treatment of many different diseases. The basic idea of gene therapy involves delivery of an exogenous gene into the cells to express the encoded protein, which may be insufficiently or aberrantly expressed naturally (Li and Huang 2006). DNA delivery is, however, a difficult process and a suitable vector is required for efficient protection as well as release. Both viral and nonviral vectors have been used for gene delivery. Nonviral gene delivery is used in DNA condensation by cationic agents. Cationic polymers have been widely chosen to condense DNA through electrostatic interactions between negatively charged DNA and the positively charged cationic sites (Mann et al. 2008). γ-PGA–CT nanoparticles have been applied for DNA delivery system. CT/DNA complex nanoparticles have been considered as a vector for gene delivery. Although advantageous for DNA

packing and protection from enzymatic degradation, CT-based complexes may lead to difficulties in DNA release at the site of action. To improve the transfection efficiency of CT/DNA complexes, γ-PGA/CT/DNA conjugated nanoparticles were prepared by an ionic-gelation method for transdermal DNA delivery using a low-pressure gene gun (Lee et al. 2008). Plasmid DNA was mixed with an aqueous γ-PGA (20 kDa). Nanoparticles were obtained upon addition of the mixed solution into an aqueous CT (80 kDa). The solutions were thoroughly mixed for 10–15 s, and then nanoparticles were collected by centrifugation. The prepared γ-PGA/CT/DNA nanoparticles were pH-sensitive and had a more compact internal structure with a greater density than the conventional CT/DNA. The analysis of small angle X-ray scattering (SAXS) results indicated that incorporating γ-PGA would cause the formation of compounded nanoparticles whose internal structure might facilitate the dissociation of CT and DNA. As compared with CT/DNA, γ-PGA/CT/DNA nanoparticles improved their penetration depth into the mouse skin and enhanced gene expression. Moreover, in addition to improving the release of DNA intracellularly, the incorporation of γ-PGA in nanoparticles markedly increased their cellular internalization (Peng et al. 2009). Taken together, γ-PGA significantly enhanced the transfection efficiency of this developed gene delivery system. The results indicated that γ-PGA played multiple important roles in enhancing the cellular uptake and transfection efficiency of γ-PGA/CT/DNA nanoparticles.

Kurosaki et al. also discovered a vector coated by γ-PGA for effective and safe gene delivery (Kurosaki et al. 2009). To develop a useful nonviral vector, PIC constructed with pDNA, polyethylenimine (PEI), and various polyanions, such as polyadenylic acid, polyinosinic–polycytidylic acid, α-polyaspartic acid, and γ-PGA, was prepared. The pDNA/PEI complex had a strong cationic surface charge and showed extremely high transgene efficiency although it agglutinated with erythrocytes and had extremely high cytotoxicity. The γ-PGA could electrostatically coat the pDNA/PEI complex to form stable anionic particles. The coating of γ-PGA dramatically decreased the toxicities of pDNA/PEI complex. Moreover, the pDNA/PEI/γ-PGA complex was highly taken by the cells via γ-PGA-specific receptor-mediated pathway and showed extremely high transgene efficiencies. Further studies would be necessary to examine the detailed uptake mechanism and clinical safety as gene delivery vector.

4 Hydrogels for Tissue Engineering

Since tissue-engineering concept was proposed in 1993 (Laner and Vacanti 1993), many trials have been performed to develop biodegradable and biocompatible scaffolds for regenerating tissues and delivery platform of incorporated drugs. A polymeric scaffold is significant not only for keeping space and stiffness for cell growth, differentiation, and organization, but also for supplying nutrients. In general, poly(lactic acid) and poly(glycolic acid) are used for preparing the scaffold, but they have a limitation such as low functionality, induction of inflammation during

hydrolysis, and hardness mismatch to the surrounding tissues. Recently, application of γ-PGA as a scaffold has attracted much attention in tissue-engineering field.

4.1 γ-PGA–Sulfonate as a Heparionid Polymer

Heparin, highly sulfated glycosaminoglycan, is known to have many biological activities, such as anticoagulant activity, antiviral activity, and plasma cleaning activity. However, heparins sometimes give rise to side effects such as hemorrhage (Leonard and Scribner 1969), thrombocytopenia (Kelton 1986), allergies, and lipid metabolism (Deuber and Schultz 1991). Accordingly, sulfated naturally occurring polysaccharides have been studied as a heparin-like polymer (heparinoid), such as dextran (Mauzac and Jozefonvictz 1984), chitin (Nishimura and Tokura 1987), and chitosan (Muzzarelli et al. 1984). However, conventional heparinoids derived from naturally occurring polymers have a limitation to control sulfate content due to the low chemical reactivity of the saccharic structure. Matsusaki et al. recently synthesized sulfonated γ-PGA (γ-PGA-S) by condensation of amine group of 2-aminoethane-1-sulfonic acid (taurine) to carboxyl group of γ-PGA (Matsusaki et al. 2002). Sulfonate contents of γ-PGA-S were easily controlled from 0 to 80% by changing concentration of condensation reagents in feed, and the anticoagulant activity increased with increasing the sulfonate content (Fig. 14).

Heparin is also known to have a stabilizing activity of growth factors as an important biological activity (Fahama et al. 1996). Basic fibroblast growth factor (FGF-2) activities of γ-PGA-S were investigated in relation to the sulfonate content by chlorate-treated fibroblast cell culture test (Matsusaki et al. 2005a). The chlorate-treated L929 fibroblasts that had suppressed biosynthesis of heparin sulfate proteoglycans (Keller et al. 1989) were used to analyze the FGF-2 activity of γ-PGA-S. When 72% of carboxyl groups in γ-PGA were sulfonated (γ-PGA-S72), cell number reached a maximum, suggesting higher FGF-2 activity. The activity of γ-PGA-S72 was higher than that of other heparinoids (Fig. 15). The γ-PGA-S72 also revealed higher FGF-2 protective effects in comparison to original γ-PGA. Molecular modeling experiment demonstrated that electrostatic interactions can take place between sulfonic and carboxylic acids of γ-PGA-S and basic amino acid residues of FGF-2. Therefore, the fairly high biological activities of γ-PGA-S would be useful to prepare biologically active scaffold for tissue engineering.

4.2 γ-PGA–Sulfonate Hydrogels with FGF-2 Activity

Tissue engineering and regenerative medicine have been directed in the development of next-generation medical technology, and three key factors – cells, growth factors, and scaffolds – are important for tissue engineering. The injection of growth factor solutions has been used to heal and regenerate tissues and organs.

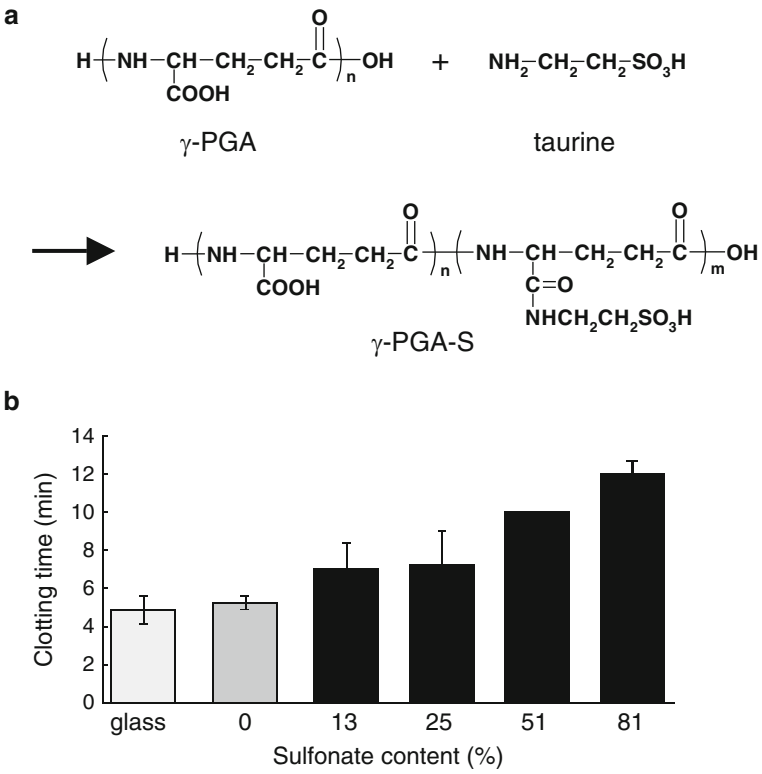


Fig. 14 Synthetic scheme of sulfonated γ-PGA (γ-PGA-S) (**a**) and effect of sulfonate content on anticoagulant activity at 1 mg/ml (**b**) (Updated from Matsusaki et al. 2002)

However, it is difficult to directly apply bioactive proteins to tissue-engineering processes because of their sensitivity to denaturation and degradation by external environmental factors such as pH and enzyme (Cohen et al. 1991). The incorporation of various growth factors into scaffolds has been actively studied, but the localized and stimulus-sensitive controlled release of growth factors from conventional hydrogels has not been achieved yet due to the primary burst and deactivation of the released growth factors. It is therefore desirable to develop hydrogels with growth factor activity that can stably interact with the hydrogel over a long time, and for the activity of the released growth factors to be maintained because the hydrogel can protect them from environmental denaturation like proteoglycans such as heparansulfate proteoglycan. Furthermore, a strategy for the stimulus-sensitive delivery of growth factors to a target area is required for localized and controlled release. Therefore, γ-PGA–S hydrogels with FGF-2 activity as a platform for stable incorporation of FGF-2 were prepared (Matsusaki et al. 2005b) and pH-sensitive release of the incorporated FGF-2 was investigated (Matsusaki and Akashi 2005). It was designed as a semi-interpenetrating polymer network (semi-IPN)-like hetero gel (S72-netgel), consisting of γ-PGA crosslinked network and

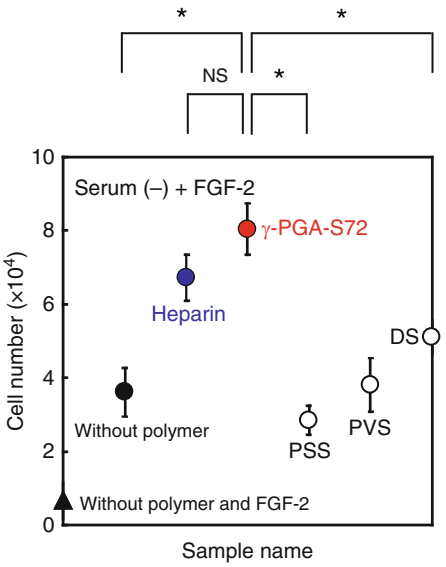


Fig. 15 Comparison of FGF-2 activity of γ-PGA-S72 to that of various heparinoids (Updated from Matsusaki et al. 2005a)

incorporated γ-PGA-S72 polymer chain, to maintain mobility and FGF-2 activity of γ-PGA-S72 (Fig. 16a). The swelling ratio and cell adhesion property of the S72-netgels increased with increasing incorporation amount of γ-PGA-S72. S72-netgels including 36 mol% sulfonic acid (S72-netgel-36) could encapsulate 280 ng of FGF-2 and retained about 60% of the FGF-2 even after 15 days of incubation in buffer, probably due to the stable interaction of FGF-2 with γ-PGA-S72 in the netgels. The release of FGF-2 from the S72-netgel-36 was successfully controlled by alternating immersion in pH = 7.4 and acidic pH buffers such as pH = 6.0 and 4.0 because of shrinkage of the gels at acidic pH condition corresponding to protonation of carboxyl anion (Fig. 16b). Furthermore, the FGF-2 released from the hydrogels retained its activity without denaturation. It has been reported that the ischemic, inflamed, and tumor areas in the body have an acidic pH (<6.5) compared to the surrounding tissues and blood (Na et al. 2004). These γ-PGA-S scaffolds might be useful as a pH-sensitive controlled delivery matrix for tissue engineering, such as the neovascular treatment of ischemia and inflammation.

4.3 Hydrogel Template Approach for Tissue Engineering

The *in vitro* construction of engineered tissues composed of cells and three-dimensional (3D) polymeric scaffolds that act as analogs to the natural extracellular matrix (ECM) has been studied as a typical tissue-engineering approach (Lee and Mooney 2001). However, polymeric scaffolds still have a risk of immunogenicity, disease transmission, or inflammation during the hydrolysis process in the

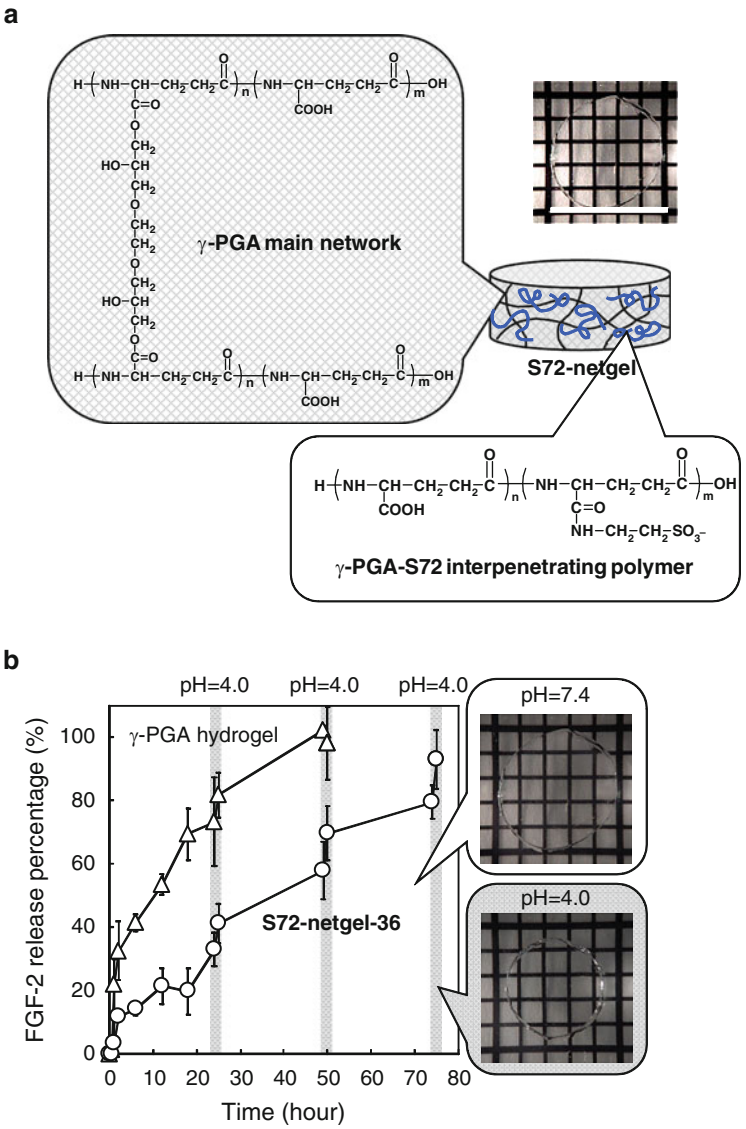


Fig. 16 Schematic illustration of a semi-IPN-like hetero gel (S72-netgel) composed of γ-PGA and γ-PGA-S72 (**a**) and pH-sensitive stepwise release of FGF-2 from S72-netgel-36 (**b**) (Updated from Matsusaki and Akashi 2005)

transplanted parts (Hubbell 2003). Since a scaffold is significant not only for keeping space and stiffness for cell growth, differentiation, and organization, but also for supplying nutrients, substitution of synthetic components of scaffold to ECM components produced from cultured cells will be useful to avoid these risks. Therefore, Matsusaki et al. designed hydrogel template approach for the *in vitro*

construction of a large-sized 3D-engineered tissue composed of cultured cells and ECM produced by the cells (Matsusaki et al. 2007). The disulfide-crosslinked γ-PGA hydrogels (γ-PGA-SS gels) were prepared as a template hydrogel. The fibroblast cells were 3D-cultured on/in the freeze-dried template gels for 10 days, and the 3D-engineered tissues consisted of the cultured cells and ECM was successfully obtained by the decomposition of the template with cysteine, because it is well known that the disulfide-crosslinked hydrogels can be decomposed by cleaving the disulfide linkage (–S–S–) to thiol groups (–HS SH–) with biocompatible reductants such as glutathione and L-cysteine (Fig. 17). The obtained engineered tissue was self-standing and highly dense composite of the cultured cells and collagen produced by the cells. Furthermore, the usage of FGF-2 and ascorbic acid can produce thicker and highly cell-incorporated engineered tissues (Yoshida et al. 2010).

Since the disulfide-crosslinked template scaffold does not be limited to a hydrogel, a nanofiber matrix is also good candidate as the template scaffold. A water-insoluble and reduction-responsive nonwoven scaffold was developed from γ-PGA (Yoshida et al. 2009a). γ-PGA nonwovens were crosslinked by cystamine with disulfide bond, and these crosslinked fibers (γ-PGA-SS fiber) were easily decomposed under physiological condition using reductant, L-cysteine. Furthermore, the γ-PGA-SS fiber revealed excellent cell adhesion and proliferation (Fig. 18). The γ-PGA-SS nonwovens can be applicable as a disulfide template matrix to this template approach.

In tissues and organs, cells and ECMs are regularly arranged to form hierarchical architectures such as the liver, skin, and blood vessels, and thus oriented 3D-engineered tissues have attracted much attention in the material science field (Hubbell 2003). To obtain 3D-engineered tissues consisting of oriented cells and ECM, pore structure of γ-PGA-SS gels by using silica cloth constructed by weaving silica fiber of 9-μm-diameter was controlled (Yoshida et al. 2009b). Since silica easily dissolves in hydrofluoric acid (HF) solution, porous structures derived from silica templates can be formed in hydrogels by removing silica with HF (Suzuki et al. 2000). The γ-PGA-SS gels with aligned 2D porous structures (OP gels) were successfully fabricated, and cell culture experiment revealed well alignment of the adhered cells on/in the OP gels. Moreover, 3D-engineered tissues composed of oriented cells and ECM produced by the cells could be obtained by decomposition of the template gels (Fig. 19). This template approach with OP gels will be powerful technique for *in vitro* construction of implantable tissue with oriented structures of cells and ECM.

5 Concluding Remarks and Future Perspectives

γ-PGA is a very promising biodegradable polymer that produces various strains of *Bacillus*. Potential applications of γ-PGA as thickener, cytoprotectant, humectant, biological adhesive, flocculant, or heavy metal absorbent, etc. have been reported.

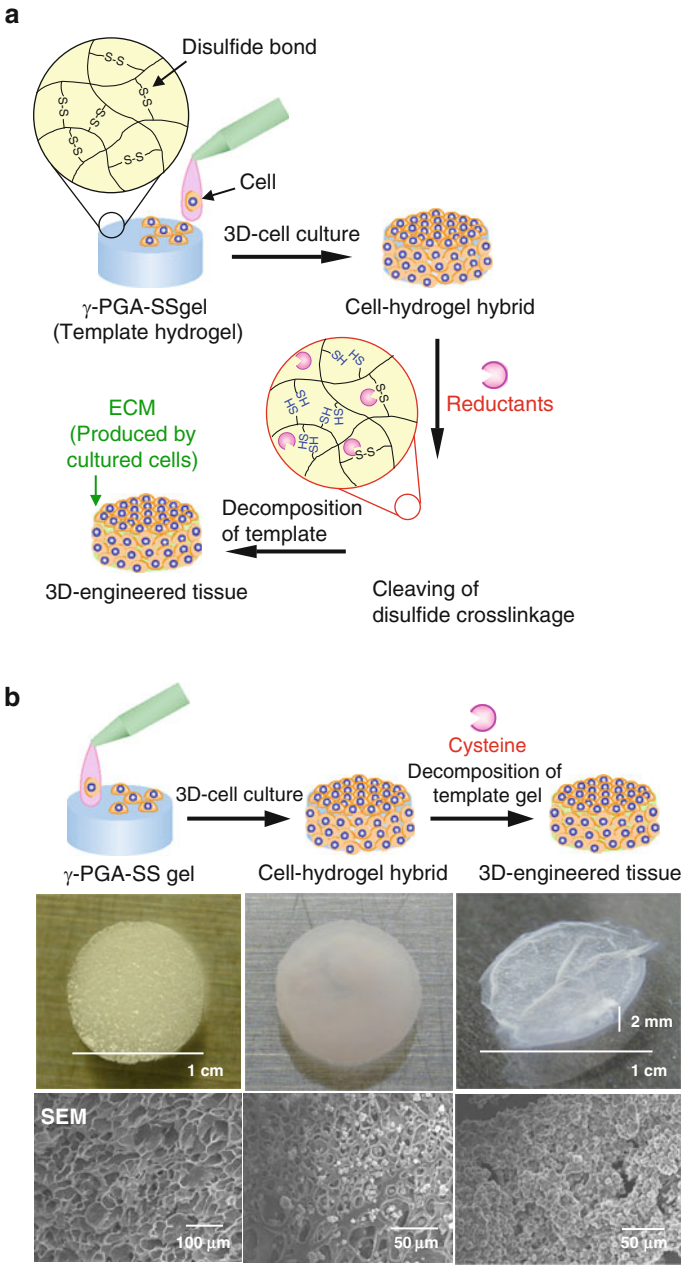


Fig. 17 Schematic representation of the 3D-engineered tissues fabricated by hydrogel template approach (**a**). Photographs and SEM images of the template gel, the cell–gel hybrid, and the 3D-engineered tissues (**b**). The template gel was decomposed in medium containing 5 mM cysteine at 37°C for 12 h (Updated from Matsusaki et al. 2007)

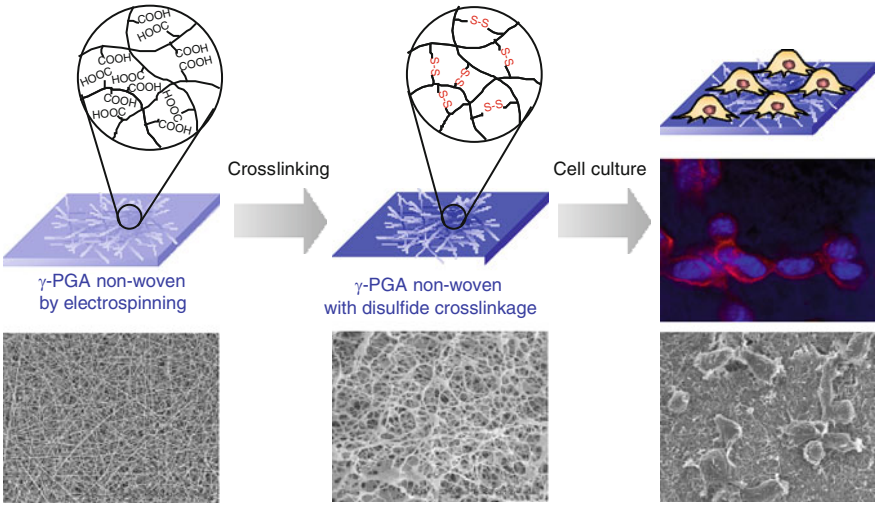


Fig. 18 Schematic illustration of the disulfide-crosslinked electrospun γ-PGA nonwovens with cell adhesive property. The disulfide-crosslinked nonwovens can be useful as a template fiber matrix to develop 3D-engineered tissue by the template approach (Updated from Yoshida et al. 2009a)

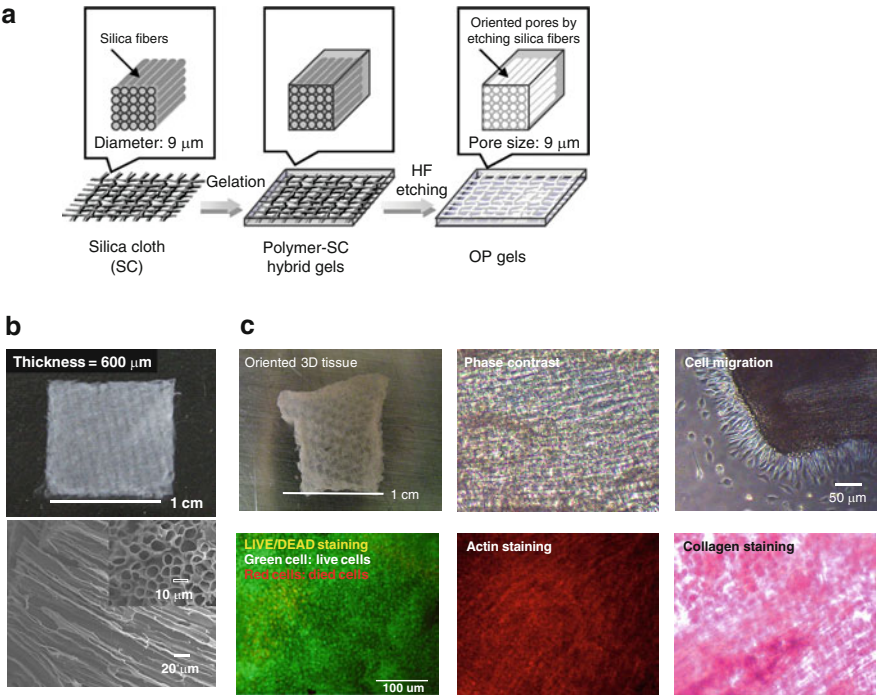


Fig. 19 Schematic illustration of the formation of OP gels (**a**). Photograph and SEM image of OP gels (**b**). Photograph and varied evaluations of the obtained 3D-tissues with 2D-orientation of cells and ECM (**c**) (Updated from Yoshida et al. 2009b)

This review describes the preparation of polymeric drugs, nanoparticles, and hydrogels composed of γ-PGA and their pharmaceutical and biomedical applications. The production of γ-PGA has already been established on the industrial scale because it can be produced easily and extracellularly in high yield by culturing of bacteria in a fermenter. Moreover, the various molecular weight of γ-PGA can be obtained commercially. γ-PGA by itself is shown to be weakly or nonimmunogenic and safety. Biomaterials consisting of γ-PGA described in this review are expected to be introduced into clinical studies in the near future.

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Food Applications of Poly-Gamma-Glutamic Acid

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Contents

Abstract Poly-γ-glutamic acid (γ-PGA) is a unique polymer in which both D- and L-glutamic acids are combined with γ-linkages. γ-PGA is safe, water soluble, anionic, and edible substance, which has many features such as high water absorbability, viscosity, metal-binding property, less susceptible for intestinal enzymes, and tasteless, etc. and the production of which has been already industrially established. Therefore, γ-PGA is highly attractive, promising food ingredient in the food industry. Several food applications using its physiological and physical properties modifying and taste perception modifying function have been demonstrated at present. However, the mechanism which γ-PGA demonstrates its

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functions and the relationships between the functions and its structural properties (molecular weight, D/L ratio, etc.) has not been understood. Detailed research in this area will be needed and clarity in this area will lead to develop more attractive applications of γ-PGA.

1 Introduction

Poly-γ-glutamic acid (γ-PGA) is well-known as a component of traditional Japanese food, natto. Natto is made from soybeans fermented by *Bacillus subtilis*. γ-PGA is a main component of natto mucilage (viscous substances) produced by *B. subtilis*. Similar products made from fermented soybeans have been also eaten in several areas in Asia: Kinema in Nepal, Thua Nao in Thailand, and Chungkookjang in Korea, etc. These traditional foods also contain γ-PGA made with *B. subtilis*. Therefore, γ-PGA has a long history of safe use in Japan as well as in the other countries in Asia as a food substance. In Japan, γ-PGA is registered as a food additive under the name of *B. subtilis* gum. Several toxicological studies including 90-day subchronic oral toxicity studies (Nakamura et al. 1999) were conducted to confirm safety of industrial γ-PGA.

γ-PGA is an unusual anionic polymer/polypeptide in which both D- and L-glutamic acids are combined with γ-linkages. In relation to the structural characteristics, γ-PGA has several features which make γ-PGA promising in the food industry. γ-PGA is biodegradable. However, γ-PGA containing D-glutamic acids and γ-linkages within its sequence is less susceptible to intestinal digestive enzymes than usual polypeptide containing L-amino acids and α-linkage. γ-PGA is water soluble and has a highly water-holding capacity. γ-PGA has a highly metal-binding ability. γ-PGA solution with high concentration has viscosity. γ-PGA containing L-glutamic acids within its sequence is tasteless compared with L-glutamic acid (especially sodium salt) having a strong taste. γ-PGA has several functions attractive for food application: e.g., physiological function, physical properties modifying function, and taste perception modifying function. The production of γ-PGA has already been established on the industrial scale because it can be produced easily in high yield. Several food applications of γ-PGA using these functions in food industries have been reported and some products have already been launched into the food market.

2 Physiological Function

2.1 Enhancing Intestinal Calcium Absorption

Calcium (Ca) absorption from the intestine is relatively low. Generally, Ca needs to be soluble in the intestine to be absorbed, but solubility of Ca in the intestine is affected by several factors. Among dietary factors, phytate and oxalate are well

known to form insoluble and unabsorbable complexes with Ca in the intestinal tract and to reduce intestinal Ca absorption of calcium. On the other hand, casein phosphopeptide (CPP) is well known to increase intestinal Ca absorption by enhancing Ca solubility owing to negative charges within its molecule. It is reported that CPP increases Ca solubility in vitro and in vivo and accelerates Ca absorption and retention in animals, and Ca absorption in humans.

Since γ-PGA has many carboxyl groups within its molecule, we expected that γ-PGA also has Ca solubilizing ability. Moreover, it was expected that γ-PGA maintains its Ca solubilizing ability even in the intestinal tract since γ-linkages are less susceptible to intestinal enzymes. Thus, γ-PGA was expected to increase Ca solubility in the intestinal tract and then intestinal Ca absorption. Actually, γ-PGA is resistant to intestinal digestive enzymes such as pepsin, trypsin, and chymotrypsin and also resistant to other proteases such as elastase, thermolysin, and pronase, etc. (Tanimoto et al. 2001b). Furthermore, we confirmed γ-PGA is only partially degraded and excreted into feces in rat feeding studies (unpublished data).

Ca solubilizing ability of γ-PGA in vitro as well as in vivo was reported by using natto mucilage, the main component of which is γ-PGA (Tanimoto et al. 2001a). In the in vivo study, both the amount and the ratio of soluble Ca in the lower part of the small intestine in rats fed with natto mucilage were significantly increased. The effect of γ-PGA on Ca absorption and retention was investigated by a metabolic balance study using rats (Tanimoto et al. 1998). In a result, Ca retention of rats was significantly increased by γ-PGA, which suggested that γ-PGA accelerates Ca absorption by solubilizing Ca in the intestinal tract. Bone Ca content of tibia in rats was also increased by γ-PGA in the study. Finally, the effect of γ-PGA on bone formation was confirmed by chick feeding study (Tanimoto et al. 1998). Bone Ca content of tibia in chick was significantly increased by γ-PGA.

Finally, the effect of γ-PGA on Ca absorption in humans was investigated. A single-blind, randomized, crossover study was performed and true fractional Ca absorption (TFCA) in 24 healthy nonsmoking postmenopausal women was examined by double isotope method using stable isotopes (^{44}Ca and ^{42}Ca) to determine the effect of γ-PGA (Tanimoto et al. 2007). In a result, γ-PGA was shown to enhance TFCA significantly and found to increase Ca absorption in humans (Fig. 1). The effect of γ-PGA was remarkable in the subgroup of the subjects whose TFCA without γ-PGA was less than the mean value (Fig. 2). It was suggested that γ-PGA had greater benefit for individuals with low Ca absorption. A Ca supplement containing γ-PGA has been developed and the stimulatory effect of the supplement on Ca absorption in humans was investigated by measuring urinary Ca excretion (Tanimoto et al. 2003). In 16 men, urinary ΔCa from the initial value of the test group (Ca supplement containing γ-PGA was given) was significantly higher than that of the control group (Ca supplement without γ-PGA was given). On the other hand, there were no differences in urinary bone resorption markers between the groups. In conclusion, the Ca supplement containing γ-PGA was suggested to increase Ca absorption in humans.

Other groups also researched the effect of γ-PGA on Ca solubility, absorption and bone formation. Ca solubilizing ability of γ-PGA in vitro as well as in vivo (in the upper and lower intestine) was reported (Park et al. 2005). They reported Ca

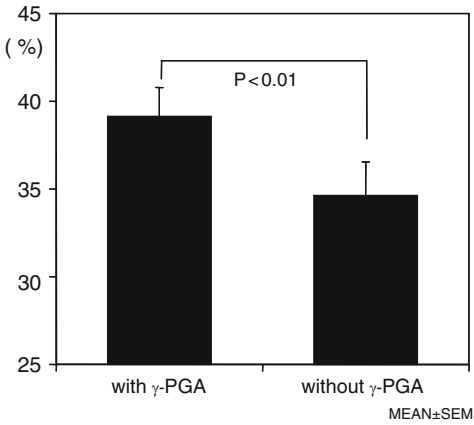

Fig. 1 True fractional Ca absorption

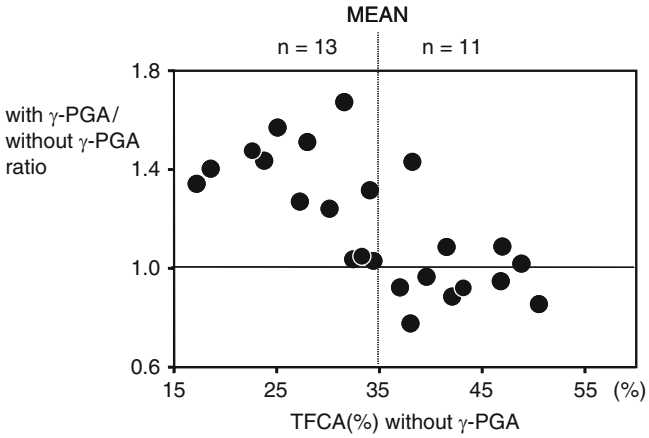

Fig. 2 Relationship between TFCA (%) without γ-PGA and ratio of ±γ-PGA

solubilizing ability in the mouse small intestine of high-molecular-weight γ-PGA (MW 1,000 K) was higher than that of γ-PGA (MW 500 K) and suggested that molecular size of γ-PGA was correlated with Ca solubility. Moreover, Ca solubilizing ability of γ-PGA in vitro was also reported (Yang et al. 2008). They conducted single oral dose study with rats and investigated intestinal Ca solubility and Ca absorption assessed by plasma Ca kinetics. In a result, γ-PGA was shown to increase Ca solubility and absorption. They also conducted a metabolic balance study using rats and confirmed that γ-PGA accelerated apparent Ca absorption, Ca retention, bone Ca content, and finally bone mineral density. However, they reported γ-PGA decreased Ca solubility in the intestinal tract in this chronic Ca-balance experiment.

It is generally accepted that intestinal Ca absorption involves two processes: a saturable (active and transcellular) transport that is regulated by vitamin D and

takes place in the upper small intestine, and a nonsaturable (passive and paracellular) transport that is dependent on soluble Ca concentration and takes place in the whole intestine but predominates in the lower small intestine. In addition, it is also generally accepted that saturable transport is dominant only at low Ca concentrations, and nonsaturable transport becomes dominant at higher luminal Ca concentrations, though there has been an argument. Therefore, γ-PGA is considered to increase nonsaturable transport, especially in the lower intestine, which is consistent with the data that γ-PGA increases Ca solubility in the lower small intestine (Tanimoto et al. 2001a; Park et al. 2005). However, it is reported that γ-PGA decreased Ca solubility when apparent Ca absorption and retention was accelerated by γ-PGA (Yang et al. 2008). Furthermore, expression of calbinding-D9k, a vitamin D-induced Ca-binding protein, in the upper small intestine was also increased in the same experiment, which suggested that γ-PGA increased saturable Ca transport in the upper small intestine. They suggested that the retardation of intestinal transit time occurred by γ-PGA in this chronic Ca-balance experiment was responsible for the increase of Ca absorption. The mechanism which γ-PGA increases Ca absorption is still unclear. Further studies will be needed to clear the issue.

2.2 *Dental/Oral Care*

The application on the use of γ-PGA as a salivation promoter (sialagogue) is demonstrated (Uotani et al. 2005a). The food composition containing γ-PGA as a salivation promoter available for chewing gum, candy, and drink is reported. Accordingly, the composition is useful in preventing dry mouth resulting in unpleasantness, halitosis, and dental caries without impairing the taste of the composition. The salivation promoting activity of γ-PGA was investigated in humans compared with physiological saline. They reported 30–50% higher salivation promoting ability of γ-PGA (average Mw of γ-PGA is 1,200 K and 300 K) than physiological saline. On the other hand, the effect of hyaluronic acid is only 10% higher than physiological saline.

The effect of γ-PGA on dental recalcification is also demonstrated by them (Uotani et al. 2005b). The food applications (e.g., chewing gum and candy) of γ-PGA effective in promoting dental recalcification and preventing dental caries are introduced. Solubilization of oral Ca is essential for dental recalcification, and dental recalcification occurs in the result of the pH adjustment of saliva. Therefore, the food applications promote dental recalcification directly through its Ca solubilizing ability and indirectly through its salivation promoting ability.

2.3 *Antidiabetic Activity*

The application on the use of γ-PGA as a hypoglycemic agent for food is reported (Uotani and Takebe 2005). They found that γ-PGA prevented increase of blood

glucose by making intestinal absorption of sugar slower. They suggested viscosity of γ-PGA made intestinal transit time slower, in a result, absorption of sugar was delayed. They confirmed this effect by investigating time courses of blood glucose level in rats after single oral ingestion of sucrose with/without γ-PGA.

The insulin-mimetic potential of vanadium is confirmed in vitro as well as in vivo in both animal and human studies. Among vanadium salts, vanadyl salts (+4 oxidation state of vanadium), e.g., vanadyl sulfate ($VOSO_4$), get attention. However, it is a problem that the absorption and incorporation of this inorganic salt is generally considered to be very low. New vanadyl compound, vanadyl-γ-PGA complex (VO-γ-PGA), has been introduced to have an antidiabetic activity (Karmaker et al. 2006). It was found that VO-γ-PGA had significantly higher in vitro insulin-mimetic activity than $VOSO_4$, which was examined by determining both inhibition of free fatty acid release and glucose uptake in isolated rat adipocytes, and that VO-γ-PGA had higher bioavailability than $VOSO_4$, which was examined by metallokinetic study in which time courses of vanadyl concentration in the blood of rats treated intravenously with VO-γ-PGA or $VOSO_4$. Moreover, VO-γ-PGA showed a significant higher hypoglycemic effect than saline in both single oral administration study and daily oral administration study for 16 days using streptozotocin(STZ)-induced Type 1 diabetic mice. In addition, the effect is higher than $VOSO_4$. Finally, antidiabetic effect of VO-γ-PGA was also supported by the results of oral glucose tolerance test using STZ-mice and HbA_{1c} level in the serum of STZ-mice after 16 days administration.

Further studies to confirm the antidiabetic activity of VO-γ-PGA were conducted by them (Karmaker et al. 2008). A longer period time (28 days) of daily oral administration study using STZ-mice showed that $VOSO_4$ (10 mg/kg BW as vanadium) had significant antidiabetic ability, blood glucose in mice treated with $VOSO_4$ improved up to the blood glucose level in nondiabetic normal standard ddY mice, and that the improvement was strongly correlated by the improvement in oral glucose tolerance ability and HbA_{1c} level. The food composition containing VO-γ-PGA as an antidiabetic agent is demonstrated (Sakurai et al. 2007).

Antidiabetic effect of Zn-γ-PGA complex (Sakurai et al. 2007) and Cu-γ-PGA complex (Karmaker et al. 2007) was also reported.

2.4 Prevention of Increase of Blood Pressure

It is reported that potassium salt of γ-PGA (K-γ-PGA) adsorbs sodium in replacement of releasing potassium (Kishimoto et al. 2008). The food composition containing K-γ-PGA which is effective for preventing and improving hypertension is demonstrated. They conducted animal study using stroke-prone spontaneously hypertensive (SHRSP) rats and investigated the effect of K-γ-PGA on an increase of blood pressure. It is found that the diet containing K-γ-PGA significantly prevented increase of blood pressure of SHR rats compared with control. Also, in K-γ-PGA group fecal sodium and urine potassium were significantly higher than those in control group. Renin–angiotensin system evaluated by analyzing serum

factors in vitro in K-γ-PGA group was significantly depressed. Moreover, plasma NOx, which was known to increase in SHRSP rats, was lower in K-γ-PGA group than in control. Accordingly, it was concluded that K-γ-PGA prevented the increase of blood pressure by reducing sodium absorption and retention, increasing potassium absorption, depressing rennin–angiotensin system, and lowering plasma NOx. The effect of $KHCO_3$ was also evaluated. $KHCO_3$ was still effective but less effective than K-γ-PGA.

2.5 Skin Care

Vitamin C is essential for collagen formation, so that is needed for repair and growth for skin tissues. Furthermore, vitamin C has antioxidation function effective in protecting ultraviolet rays and preventing pigmentation of skins. However, vitamin C is very unstable physicochemically and easily broke down by heat, light, etc. It is reported that γ-PGA–vitamin C complex in which carboxyl group of γ-PGA links with hydroxyl group of vitamin C by ester bond improves the stability of vitamin C which plays an important role for skins (Sung et al. 2008). They also reported increased intestinal absorption of vitamin C when the complex was orally administered with mice.

Moreover, they found that γ-PGA–vitamin C complex has an inhibitory effect on collagenase, an enzyme which degrades collagen present in skins and reduces skin elasticity and form wrinkles. (Sung et al. 2007). The food composition containing γ-PGA–vitamin C complex as an inhibitor of collagenase is demonstrated. As skin aging progresses, the expression of collagenase is promoted. Also, collagenase is activated by ultraviolet irradiation. By inhibiting skin collagenase activity the composition is effective for improving skin elasticity and reducing wrinkles. Therefore, the composition is effective for skin not only by inhibiting collagenase activity, but also by having antioxidant effect leading to antiaging of skin. In the result of collagenase inhibition assay in vitro they conducted, γ-PGA–vitamin C complex had an inhibitory effect on collagenase. On the other hand, neither just a mixture of γ-PGA and vitamin C nor vitamin C alone had any effect on collagenase activity. In addition, antioxidant effect of γ-PGA–vitamin C complex assessed by free radical removing ability in vitro was as high as that of vitamin C.

The functional food containing γ-PGA for preventing or improving atopic dermatitis by normalizing immune system imbalances is also introduced by them (Sung et al. 2009b).

2.6 Other Functions

The food composition for preventing thrombus formation which contains γ-PGA as an anticoagulant is reported (Sung et al. 2009c). The anticoagulant effect of γ-PGA

was investigated in vitro using blood of mouse. As a result, γ-PGA had an anticoagulant ability at a concentration of 0.5% in an MW range of 50–10,000 K regardless of MW. When the anticoagulant ability of γ-PGA was compared with that of heparin, γ-PGA had similar ability to heparin at a concentration of 5 mg/ml.

The food composition for treating or preventing hypertriglyceridemia which contains γ-PGA as an inhibitor of lipid absorption is demonstrated (Nogusa et al. 2009). It is found that γ-PGA inhibit lipase activity in vitro. In animal studies using both rats and mice, the inhibitory effect of γ-PGA on lipid absorption was confirmed by time courses in serum triglyceride level after a single oral ingestion of lipid with/without γ-PGA.

The application on the use of γ-PGA as a blood ammonia lowering agent (Hidesaki and Tanimoto 2007a) or as a bowel moving improver (Hidesaki and Tanimoto 2007b) is also demonstrated. Finally, the functional food containing γ-PGA as an effective ingredient for inhibiting viral infection to prevent diseases is also introduced (Sung et al. 2009a).

3 Physical Properties Modifying Function

3.1 Antifreeze Activity

Freezing and thawing frequently cause undesirable deterioration in foods. To protect them from deterioration, the addition of cryoprotectants has been widely used. It is reported that sodium salts of peptides mainly consisting of acidic amino acids (e.g., enzymic digests of bovine hemoglobin) or sodium salts of acidic amino acids (e.g., monosodium glutamate) have high antifreeze activity. According to these results, antifreeze activities of various types of polyglutamic acids (γ-PGAs and poly-α-glutamic acids) were investigated by differential scanning calorimetry (DSC) to characterize their antifreeze activities and clarify the relationships between their antifreeze activities and chemical structures (Mitsuiki et al. 1998). As a result, the antifreeze activities of PGA tended to decrease as their molecular weights increased; however, PGA with molecular weight range below 20 K had antifreeze activity higher than that of glucose, which is known as a highly antifreeze substance. In addition, the antifreeze activities were only slightly affected by the optical isomerism (D- or L-Glu) and the peptide linkage type (α- or γ-Iinkage) and decreased in the order Na salt = K salt >> Ca salt >> acidic form. It was suggested that the high antifreeze activities of PGA are mainly caused by Coulomb's force of the dissociating counter ions. It is believed that γ-PGA is an effective cryoprotectant for frozen foods, because it has weaker taste than the commonly used lower molecular weight cryoprotectants such as saccharides, inorganic salts, and amino acids, and therefore, it can be added to foods in larger quantities without a serious change in the taste.

Similar study in which the antifreeze activities of various types of PGAs were investigated by DSC is reported (Shih et al. 2003). They reported similar results in

which the antifreeze activities of γ-PGA increased as its molecular weight decreased but was indifferent to its D/L-glutamate composition. However, the antifreeze activities decreased in the order Mg salt >> Ca salt = Na salt >> K salt, which was different from the previous report. The mechanism by which the antifreeze activities of γ-PGA can be explained is still yet to be determined.

The application of γ-PGA in the baking industry, the use of γ-PGA as a cryoprotectant for frozen dough, is demonstrated (Yokoigawa et al. 2006). They examined the effect of γ-PGA on the freeze-tolerance of several types of commercial baker's yeast and as a result, confirmed improvement of the freeze-tolerance. The survival ratio of ordinary baker's yeast cells frozen at −30°C for 3 days was improved (10% to more than 70%) by adding more than 1% γ-PGA to the medium, irrespective of the difference of molecular weight of γ-PGA (50–10,000 K). Similar results were also obtained with other types of baker's yeast (e.g., low-temperature-sensitive yeast). When the baker's yeast cell was frozen at −30°C for 3 days in dough supplemented with more than 1% γ-PGA, the cell after freezing and thawing showed higher leavening ability than that frozen in dough without γ-PGA, irrespective of molecular weight of γ-PGA (50–10,000 K). It is concluded that γ-PGA protects baker's yeast from lethal freeze injury, as a result, the yeast cell has a high leavening ability after freezing and thawing. The application of γ-PGA for frozen foods is also demonstrated (Mitsuiki et al. 1996). By addition of γ-PGA to frozen foods, both freezing and thawing time are shortened so that unfreezable water content of frozen foods is increased.

3.2 Edible Hydrogel/Capsule

It is reported that biodegradable crosslinked γ-PGA hydrogel with a very high water absorption capacity can be prepared by γ-irradiation of aqueous solution of γ-PGA (Kunioka 1993). The specific water content of this hydrogel is as high as 3,500 (weight of water/dry weight of polymer). However, this hydrogel cannot be used in food industry since it is exposed to γ-irradiation. Edible water-absorbable polymer, prepared by metal crosslinking between γ-PGA and aluminum potassium sulfate and available in food industry, is introduced, the specific water content of which is as high as 250 (Hara 2007).

The use for edible capsule shell sheet is reported. The edible capsule prepared from nonanimal materials including γ-PGA is demonstrated (Furuta et al. 2005). The edible gelatin capsule, formed of a sheet composition containing gelatin and γ-PGA, is also reported. This gelatin capsule inhibits an insolubilization of the capsule sheet which occurs during storage of the ordinary gelatin capsule (Furuta 2005). Finally, the edible capsule prepared from a composition containing vegetable polysaccharides and γ-PGA is reported (Satokawa et al. 2008). The capsule has sufficient strength and elongation, superior adhesiveness and transparency, and a low burden on the drying step in the production process.

3.3 Other Functions

Several other applications of γ-PGA useful for food industry are reported in the patents. The use of γ-PGA as a thickener in foods/beverages is introduced. For example, the use in beverages to enhance viscosity and to improve drinkability is demonstrated (Yamanaka and Kikuchi 1990). The addition of γ-PGA in the production of starch foods, such as bakery products and noodles, to prevent aging and to improve textures and shape-retaining capacity is reported (Konno et al. 1989a). The use in cereals results in improving textures (Konno et al. 1989c).

Also, there are several reports on the use of γ-PGA as a stabilizer. For example, the uses for ice cream stabilizer (Wada et al. 1972) and foam stabilizer of egg white (Kasubuchi et al. 2008) are reported. Used in polyphenols-containing beverages (Konno et al. 1989b), γ-PGA is reported to prevent precipitation of polyphenols. Used in acidic milk beverages (Yurikusa et al. 2007) and protein-containing acidic residue foods/beverages (Yurikusa et al. 2008), γ-PGA is reported to improve protein dispersibility, thus, to prevent protein precipitation. Also, it is demonstrated that γ-PGA improves dispersibility or solubility of powdered foods and drinks into liquid (Ono et al. 2007). Dispersibility or solubility of thickener into liquid, such as xanthan gum, guar gum, pectin, and carrageenan, is also effectively improved by addition of γ-PGA (Satokawa 2009). Finally, oxidative stability of oil in water type emulsified foods, such as mayonnaise and dressings, is improved by addition of γ-PGA (Nakanishi and Tanimoto 2007).

4 Taste Perception Modifying Function

4.1 Masking Taste

Salt, a substance consisting of sodium and chloride, is essential for the maintenance of health, and appropriate amounts of salt enhance the taste of many dishes as well. But too much sodium can be harmful, like many other dietary components. Excessive intake of sodium is not only a primary cause of hypertension, which may lead to cardiovascular diseases such as stroke, heart attack, heart failure, etc., but also is associated with increased renal disease and risk of bone demineralization. The World Health Organization has set a worldwide target of reducing salt to 5 g/day or less for all adults, while actual salt intake in most developed countries remain 9–12 g/day. Efforts to reduce salt intake are made by individuals as well as food manufacturers, by using smaller amounts of salt and/or by using salt substitutes. In using either approach, taste is usually an issue and it is difficult to continue a low sodium diet for a long time.

Table 1 The effect of γ-PGA on masking the bitter taste of potassium chloride

γ-PGA	0	0.0005	0.001	0.0015	0.002	0.0025	0.003	0.0035	0.004	–
NaCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.0
KCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0
Saltiness	++	++	++	++	++	++	++	++	++	+++
Bitterness	+++	$++_{+}$	++	$+_{+}$	$+_{+}$	$+_{+}$	$+_{+}$	$+_{+}$	$+_{+}$	–

Potassium chloride is a common salt replacer. It has a salty taste in itself, but its metallic bitter aftertaste is a problem and deterrent to its use. Therefore, a common type of salt substitute with potassium chloride is a mixture of sodium chloride with a ratio of up to half and half. As this common type of salt substitute has very similar saltiness to sodium chloride, the mixture may be used just as regular salt. However, undesirable bitter taste of potassium chloride is still a problem for using this common type of salt substitute as regular salt.

It is found that γ-PGA has the effect of masking the bitter taste of potassium chloride and by using γ-PGA a palatable low(half)-sodium salt with potassium chloride can be made (Sato et al. 2008). The effect of γ-PGA on masking the bitter taste of potassium chloride was evaluated by sensory test. After preparing 1.0% mixture solution with sodium chloride and potassium chloride in the ratio of 1:1 and then adding γ-PGA at levels from 0.05 to 0.40% to the mixture, the bitter metallic taste is sensory evaluated. In result, at the rate of more than 0.15% the bitter metallic taste masked effectively (Table 1).

The use of γ-PGA as a bitterness-relieving agent is shown (Sonoda et al. 2000). The bitter taste of amino acids, peptides, minerals, vitamins, and caffeine is greatly relieved by addition of γ-PGA. It is also demonstrated that the endurance of sweet aftertaste of high-intensity sweetener such as aspartame and sucralose is inhibited and taste balance of high-intensity sweetener is improved by addition of γ-PGA (Sato and Koyama 2009). In addition, bitter aftertaste high-intensity sweetener is also inhibited by γ-PGA.

4.2 Enhancing Taste/Flavor

The use of γ-PGA as a flavor improver for spice-containing foods and beverages is demonstrated (Ishii et al. 2008). It is shown that γ-PGA improves the spiciness, prevents the deterioration in flavor, and keeps a well-balanced flavor in the food and beverages such as curry, potage soup, barbeque sauce, cola, and ginger ale. It is also shown that γ-PGA enhances body and thickness of beverages containing milk ingredients and polyphenols, such as cocoa, milk coffee, and milk tea (Ishii and Tomiyama 2007). Finally, γ-PGA strengthens the saltiness of foods and drinks such as Chinese noodle soup, in case pH of the foods and drinks is between 4.5 and 8 in taking (Kobayashi et al. 2007).

5 Conclusion and Outlook

γ-PGA is highly attractive, promising food ingredient in the food industry. Several food applications using its physiological and physical properties modifying and taste perception modifying function have been demonstrated at present. Ca supplement containing γ-PGA which increases Ca absorption and low-sodium salt with potassium chloride containing γ-PGA which masks the bitterness of potassium chloride have already been in the market. However, the mechanism which γ-PGA demonstrates its functions and the relationships between the functions and its structural properties (molecular weight, D/L ratio, etc.) has not been understood. Detailed research in this area will be needed and clarity in this area will lead to develop more attractive applications of γ-PGA.

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