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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

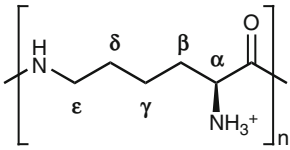

Fig. 1 Chemical structure of ε-poly-L-lysine (ε-PL) biosynthesized in microorganisms

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.

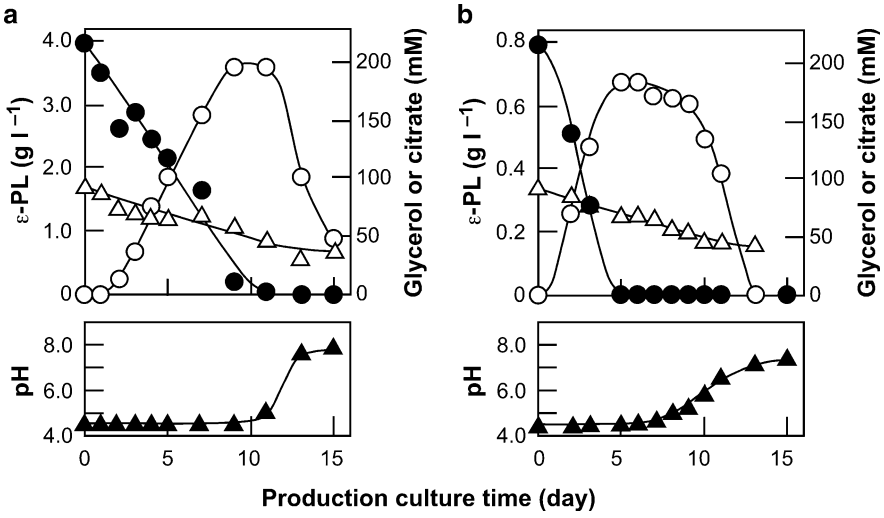

Fig. 2 Time course of ε-PL production (*open circle*), concentration of glycerol (*filled circle*) and citrate (*open triangle*), and pH of the culture medium (*filled triangle*) in: (**a**) USE-11 and (**b**) USE-51. The culture medium consisted initially of 76 mM $(NH_4)_2SO_4$ and 11 mM L-lysine·HCl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

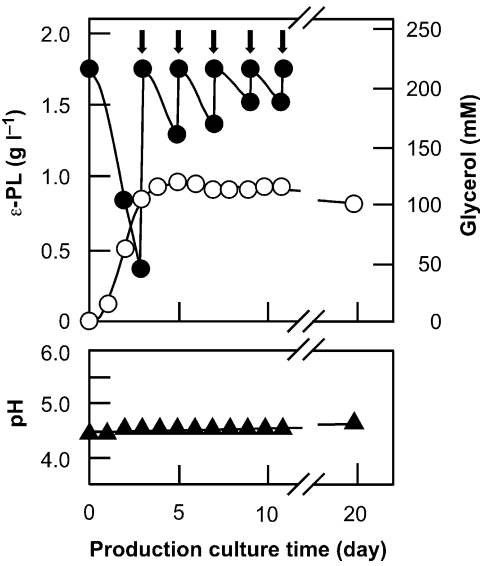

Fig. 3 ε-PL production (*open circle*), glycerol concentration (*filled circle*) and pH of the culture medium (*filled triangle*) in fed-batch culture of USE-51 cells growth-cultured for 25 h. *Arrows* indicate the addition of glycerol to maintain a concentration of 220 mM (Hirohara et al. 2006 (ESM-1))

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

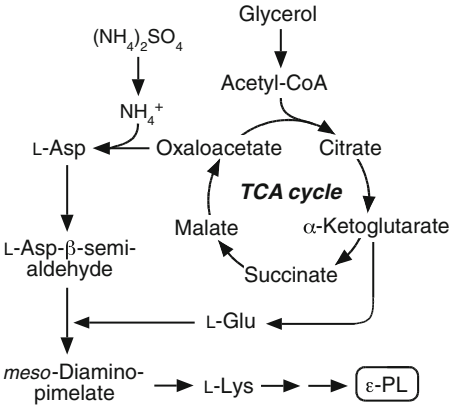

Fig. 4 Schematic representation of the putative pathway for ε-PL synthesis in *Streptomyces* strains

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.

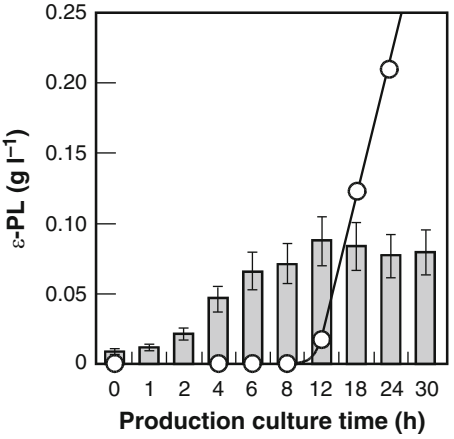

Fig. 5 Time course of ε-PL accumulation in the cells (*filled box*) and culture broth (*open circle*) of USE-11 (Saimura et al. 2008)

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

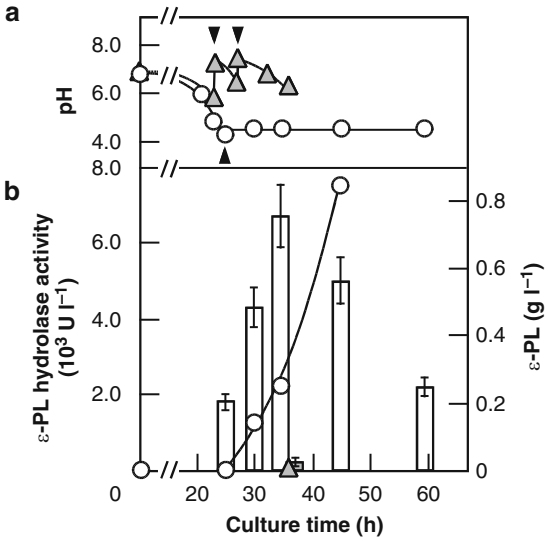

Fig. 6 Time courses of pH of the culture media, ε-PL productions at pH 4.5 (*open circle*) and pH 6–7 (*filled triangle*), and ε-PL hydrolase activity at pH 4.5 (*open box*) and pH 6–7 (*filled box*) in USE-11 during a one pot fermentation process. (**a**) The pH value decreased spontaneously to produce 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

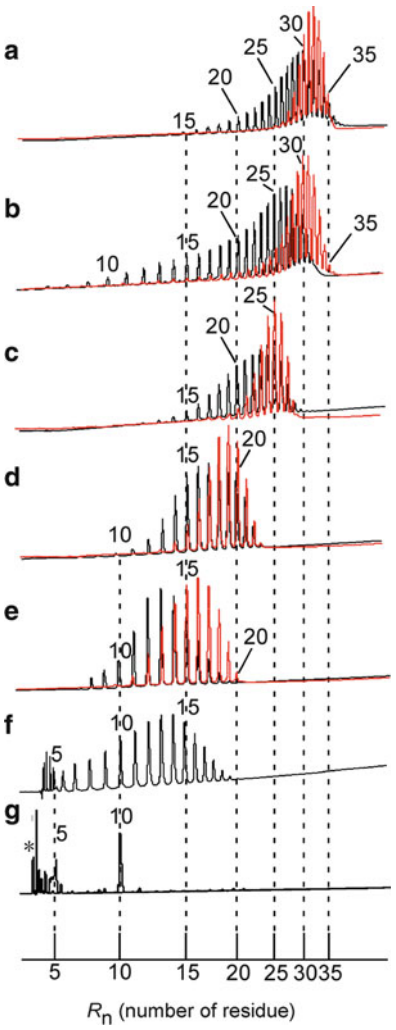

Fig. 7 Ion-pair chromatograms from the HPLC analysis of ε-PL hydrochlorides (ε-PL·HCls) produced from glucose (*red*) or glycerol (*black*). The polymers were produced by the strains: (**a**) USE-11, (**b**) USE-33, (**c**) USE-31, (**d**) USE-51, (**e**) USE-81, (**f**) partially hydrolyzed ε-PL·HCl 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