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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Substantially monodispersed poly(ε-L-lysine)s frequently occurred in newly isolated strains of *Streptomyces* sp.

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Abstract The presence of poly(ε-L-lysine) (ε-PL) was found quite frequently by screening various strains of *Streptomyces* sp. Most of the ten newly obtained ε-PLs, when they were produced from glucose, showed a polydispersity index of M_w/M_n= 1.01 using ion-pair chromatography analysis. The polymers were classified into five groups according to their chain lengths. The average numbers of residues in the five groups were 32, 28, 25, 19, and 16, respectively. The use of glycerol instead of glucose resulted in decreases of 10 to 20% in the M_n and slight increases in the M_w/M_n. These observations indicated the chain length and polydispersity of ε-PL were primarily determined by each producer strain. Proton and ^{13}C NMR analysis revealed the signals of glycerol-derived ester at the C terminus of the polymer from several producers including the first discovered *S. albulus* strain, although the percentages of the ester were low under our culture conditions. These results, coupled with the previous observation that SO_4^{2-} was essential for the polymer production, led to discussion on the mechanistic aspects of monomer activation, elongation, and termination in the biosynthesis of ε-PL.

Keywords Biocompatible · Biosynthesis · Molecular weight distribution · Poly(ε-L-lysine) · Screening · *Streptomyces* sp.

Introduction

Poly(ε-L-lysine) (ε-PL) is an L-lysine homopolymer biosynthesized nonribosomally and secreted into culture medium. It has a unique structure linking ε-amino and α-carboxylic acid functional groups. This biodegradable and highly water-soluble polymer is nonmutagenic and practically nontoxic in acute, subchronic and chronic feeding studies in rats (Hiraki et al. 2003). Furthermore, it exhibits various biological and chemical functions such as an antiphage action (Shima et al. 1982), an immunoglobulin production stimulatory effect (Maeda-Yamamoto et al. 1995), an endotoxin-selective removal action (Hirayama et al. 1999), and antiobesity action due to the inhibition of pancreatic lipase (Tsujita et al. 2003, 2006). Recently, various potential applications of ε-PL were reviewed (Shih et al. 2006). Because of its antimicrobial activity, the polymer consisting of 25–35 lysine residues is now manufactured at the commercial scale as a food preservative using a mutant of the first discovered strain of *Streptomyces albulus* No 346 (Yoshida et al. 2003; Yoshida and Nagasawa 2003; this strain is now available as *S. albulus* NBRC 14147).

A certain function of a polymeric compound depends upon its molecular weight. As a food additive, oligomeric ε-PL with ten or more lysine residues is desirable because it does not have a bitter taste and shows optimum antimicrobial activity (Shima et al. 1984). Chemically synthesized, high molecular weight ε-PL was reported to have antitumor activity (Szókán et al. 1997). Thus, it is attractive to have a wide variety of novel ε-PLs with various molecular weights. Quite recently, a quarter century after the discovery of the first strain No 346, several microorganisms were found to produce ε-PLs. Most of the polymers obtained had nearly the same chain lengths as the first discovered polymer, and some had much shorter ones (Kito et al. 2002b; Nishikawa and Ogawa 2002; Hirohara et al. 2006). Aliphatic hydroxy-compounds including glycerol were found to reduce the molecular weight of ε-PL by

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acting as chain transfer agents (Nishikawa and Ogawa 2006), as was reported previously for poly(3-hydroxybutyrate) P(3HB) (Taidi et al. 1994; Madden et al. 1999; Ashby et al. 2002, 2005).

The term ε-PL implies a mixture of polymers within a certain range of lysine residues. However, definite molecular weight distributions have not been reported for the polymers obtained. The polydispersity index (M_w/M_n) of 1.14 for the ε-PL produced by the first *S. albulus* strain is the only one reported thus far (Lee et al. 1991). This value, determined by light scattering (for M_w) and gel permeation chromatography (for M_n), was somewhat puzzling and caused speculation on the activation mechanisms of lysine for the biosynthesis of ε-PL (Nishikawa and Ogawa 2006). An accurate determination of M_w/M_n might cast new light on the mechanism of ε-PL biosynthesis. Two possible mechanisms on the activation of amino acids were known for peptide biosynthesis: AMP-forming adenylation by nonribosomal peptide synthetases (NRPSs) and ADP-forming phosphorylation by amide ligases. The former usually produces small, fixed-length peptides such as surfactin or gramicidin S (Finking and Marahiel 2004). Kawai et al. (2003) suggested from the results of ε-PL synthesis and lysine-dependent AMP formation in a cell-free system that L-lysine was adenylated in the first step of ε-PL biosynthesis. The latter produces polydispersed peptides with higher molecular weights such as poly(γ-glutamic acid) (γ-PGA; Ashiuchi and Misono 2003) or cyanophycin (CGP; Oppermann-Sanio and Steinbüchel 2003), the other two naturally occurring poly(amino acid)s well-known thus far. Kito et al. (2002a, b, 2003) used ion-pair chromatography to analyze the lengths of lysine polymers partially degraded by ε-PL-hydrolyzing enzymes. The detection of various lengths of polymers was based on the number of charged amino groups in this analysis. The careful application of this method while maintaining baseline separation would provide reliable information concerning not only the number of lysine residues (R_n) but also the relative height of each peak (individual R_n), which will enable an accurate estimation of the molecular weight and polydispersity index of ε-PL.

In this paper, we describe the occurrence of ε-PLs and their chemical structures. In the light of our best knowledge, this is the first report on the detailed molecular weights and polydispersity indexes of the polymers frequently occurred in the strains of *Streptomyces* sp. The results lead to the discussion on the possible biosynthesis mechanism of ε-PL.

Materials and methods

Chemicals

Chemically synthesized ε-L-lysine oligomers consisting of five or ten residues were a gift from Chisso (Tokyo, Japan), and were used as HCl salts. $NaClO_4 \cdot H_2O$ was obtained from Merck (Darmstadt, Germany). The L-lysine · HCl, L-lysine·2HCl, sodium octane sulfonate, and all other reagents employed in this study were purchased from commercial sources (analytical grade). The *S. albulus* NBRC 14147 was previously obtained from the Institute for Fermentation, Osaka, Japan and stocked in this laboratory.

Screening study for ε-PL-producing strains

Using the two-stage culture method of cell growth and ε-PL production cultures previously described (Hirohara et al. 2006), a screening study for novel ε-PL-producing strains was performed with a total of 1,300 (the rest of the 1,000 previously reported plus 900 newly isolated) actinomycete colonies from soil samples collected mostly in the Shiga region of Japan after the discovery and biosynthetic study of the two strains USE-11 and USE-51 (Hirohara et al. 2006) from 600 colonies. The cells were growth-cultured for 24 h and then production-cultured for 3 to 5 days. Some of them produced a trace of polycations and were again growth-cultured for 48 h followed by production culture. The culture broths were subjected to the Methyl Orange (MeO) precipitation test (Itzhaki 1972). Those strains that produced greater amounts of polycations in the broths were selected from the colonies that gave positive results on the test. Their polymers were subjected to SDS-PAGE to determine the approximate molecular sizes by comparing the migrations of broad bands of polycations against those of the polymers by strains USE-11 or USE-51. In this study, eight strains (USE-12, USE-13, USE-31, USE-32, USE-33, USE-52, USE-81, and USE-82) were chosen to investigate the chemical structures of the polymers they produced, together with those from the strains USE-11 and USE-51. The confirmation of the polycations being from ε-PLs was made by ^{1}H NMR spectra recorded on a JEOL FT NMR spectrometer at 400 MHz. The L-isomer of the lysine residues was confirmed by chiral HPLC on a Crownpak CR (+) column as previously described (Hirohara et al. 2006).

Production and purification of ε-PL

The two-stage culture method was also used for ε-PL production under the same fermentation conditions of a 2% (w/v) carbon source as previously described (Hirohara et al. 2006). The one ε-PL-producing strain USE-81 was cultured without citrate in production medium as ε-PL production was inhibited by citrate in this particular strain. The cell density-dependent producer strains USE-51, USE-52, and USE-81 were growth-cultured until the specific time that gave the highest production level in each strain. The

production cultures were continued until each strain showed the optimum production levels as determined by the MeO method.

ε-PL in the culture filtrate was purified and isolated as a white powder of ε-PL·HCl using procedures similar to ones previously reported (Hirohara et al. 2006), i.e., solvent precipitation, cation-exchange chromatography, ultrafiltration, and precipitation by acetone addition. The difference is that the method applied here omits the second ion-exchange chromatography step and active carbon treatment.

Measurement of the number of residues

The R_n of ε-PL was measured by ion-pair HPLC as per the previous analysis of the lengths of partially degraded ε-PL (Kito et al. 2002a, b, 2003) with some modifications. The measurement was performed with a JASCO GULLIVER series HPLC equipped with a TSKgel ODS-80Ts column (4.6×250 mm; Tosoh, Tokyo) equilibrated with 45% (v/v) Buffer A (10 mM NaH_2PO_4, 100 mM $NaClO_4·H_2O$, 10 mM sodium octane sulfonate, pH 2.6) and 55% Buffer B (20 mM NaH_2PO_4, 200 mM $NaClO_4·H_2O$, 20 mM sodium octane sulfonate, pH 2.6, 50% (v/v) acetonitrile). The ratio of Buffer B to Buffer A was changed in a linear gradient from 55 to 78% over 70 min at 50°C. The gradient baseline was maintained as straight as possible. The absorbance of the effluent was recorded at 215 nm. Chemically synthesized ε-lysine oligomers·HCl (R_n=5 or 10), L-lysine·2HCl, and partially hydrolyzed ε-PL·HCl (6 M HCl, 80°C, 90 min) from the strain USE-82 were used as standards for the R_n. All of the experiments were replicated at least three times.

NMR analysis of glycerol-derived ε-PL

Proton and ^{13}C NMR spectra were recorded on a JEOL JNM-ECA600 FT NMR spectrometer at 600 and 150 MHz with the number of scans being 120 and 20,000, respectively, to examine the presence of ε-PL-glycerol ester in polymer. Proton/^{13}C HMQC and HMBC spectra in the two-dimensional experiments were obtained with the scans of 120 and 160 times, respectively. Before the NMR measurements, the purified ε-PL·HCls mentioned above were dissolved in distilled water and precipitated with ethanol, and then prepared as ca. 4% solutions in D_2O containing a small amount of deuterated TMSP [3-(trimethylsilyl)propionic acid sodium salt] to perform ^1H and ^{13}C NMR experiments. The ^1H and ^{13}C NMR chemical shifts in parts per million (ppm) were measured relative to TMSP at 25°C and pD 2.7 with 5-mm diameter tubes.

Evaluation of molecular weight

The M_n and M_w of ε-PL were calculated from the results of the HPLC ion-pair chromatogram as follows:

$$M_n = \sum_i H_i \Big/ \sum_i (H_i/M_i) \quad (1)$$

$$M_w = \sum_i H_i M_i \Big/ \sum_i H_i \quad (2)$$

$$M_i = (146.19 - 18.02)R_i + 18.02 \quad (3)$$

where H_i and M_i are the height and the molecular weight, respectively, of the peak i corresponding to the individual number of residues (R_i) in the chromatogram. The values of 146.19 and 18.02 represent the molecular weight of the lysine residues and H_2O, respectively.

Identification and deposition of the strains

A taxonomic identification of the eight ε-PL-producing strains USE-12, USE-13, USE-31, USE-32, USE-33, USE-52, USE-81, and USE-82 were from the DSMZ (Braunschweig, Germany). All of them belonged to the genus of *Streptomyces* (Table 1). They were deposited in the collection of the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Tsukuba, Japan) as strains FERM P-17998, P-18561, P-19660, AP-20901, AP-20902, P-17845, P-18305, and P-19661, respectively.

Results

Occurrence and identification of ε-PL-producing strains

The screening targets were focused on the group of actinomycetes as previously described (Hirohara et al. 2006). Of the 1,300 actinomycete colonies examined, 200 colonies were found to give positive results on the MeO precipitation test. All the secretes 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 (2.5 to 4.5 kDa), although any substance having more than three cationic sites in the molecule formed insoluble complexes with MeO (Hirohara et al. 2006). No microorganisms producing ε-PL had been isolated since the first discovery of the *S. albulus* strain a quarter century ago until quite recently. It is surprising to note that the occurrence of ε-PL was much more frequent than had been

Table 1 Characterization of ε-PLs produced from glucose or glycerol[a]

Producer strain	R_n[b]		M_n[c]		M_n/M_n[c]		Production level from glycerol (g l^{-1})	Percentage of glycerol ester at C terminus[d] (%)
	Glucose	Glycerol	Glucose	Glycerol	Glucose	Glycerol		
Streptomyces lydicus USE-11	24–36	15–35	4,050±10	3,500±60	1.01	1.03	4.0	9±2
Streptomyces sp. USE-12	22–35	14–35	3,920±10	3,500±80	1.01	1.03	2.0	–
S. albulus subsp. USE-13	–	10–36	–	3,500±20	–	1.03	2.5	–
S. albulus NBRC 14147	22–36	14–35	3,960±10	3,450±60	1.01	1.05	2.8	15±3
Streptomyces sp. USE-33	12–35	8–32	3,600±30	2,920±50	1.04	1.06	0.4	15±3
S. celluloflavus USE-31	17–29	12–29	3,140±10	2,840±60	1.01	1.03	0.8	–
S. celluloflavus subsp. USE-32	–	10–29	–	2,720±70	–	1.03	0.5	10±2
Streptomyces sp. USE-51	13–23	10–23	2,390±10	2,150±10	1.01	1.03	0.8	0
S. herbaricolor USE-52	13–23	10–23	2,390±10	2,150±30	1.01	1.03	0.5	–
S. lavendulae USE-81	10–20	8–19	2,000±10	1,670±20	1.02	1.03	0.8	–
S. aureofaciens USE-82	10–21	5–20	2,080±20	1,660±20	1.02	1.06	4.0	0

[a] The production culture medium initially consisted of 2% glucose or 2% glycerol, 95 mM citrate, 76 mM $(NH_4)_2SO_4$, and 11 mM L-lysine
[b] The number of residues taken from ion-pair chromatograms
[c] Evaluated through the procedure described in the Materials and methods
[d] Estimated from the relative integrated areas of peaks of ester α-proton to those of N terminus ε-protons (see Fig. 2 for peak assignment)
– Not determined

anticipated, even if we took into account that the two-stage culture screening method was effective and the MeO detection method was highly sensitive to ε-PL. Among the 200 colonies, nearly 50 strains secreted fairly large amounts (≥0.3 g l^{-1}) of ε-PL in their culture broths. Thus, it may be safe to say that it is not difficult to obtain a good ε-PL producer from actinomycete strains.

The eight strains in this study were identified as follows: a new *Streptomyces* species, *S. albulus* subspecies, *S. celluloflavus*, *S. celluloflavus* subspecies, a new *Streptomyces* species, *S. herbaricolor*, *S. lavendulae*, *S. aureofaciens*, and the designated *Streptomyces* species USE-12 (USE-12), *S. albulus* subsp. USE-13 (USE-13), *S. celluloflavus* USE-31 (USE-31), *S. celluloflavus* subsp. USE-32 (USE-32), *Streptomyces* species USE-33 (USE-33), *S. herbaricolor* USE-52 (USE-52), *S. lavendulae* USE-81 (USE-81), and *S. aureofaciens* USE-82 (USE-82), respectively. This is all shown in Table 1 together with *S. lydicus* USE-11 (USE-11), *Streptomyces* species USE-51 (USE-51), and *S. albulus* NBRC 14147 (NBRC 14147). USE-11 and USE-51 were previously identified and designated (Hirohara et al. 2006).

Monodispersity and controlled molecular weight

The R_n obtained directly from the chromatograms, the M_n and M_w/M_n of the ε-PLs from glucose or glycerol in the eight newly isolated strains were summarized along with USE-11, USE-51 and NBRC 14147 in Table 1. This is the first determination for the polymers from these three strains as well. The ε-PLs produced can be classified into five groups according to their chain lengths. Typical chromatograms were presented together with that of the standard 5- and 10-mers in Fig. 1. It should be noted that the horizontal axis of the Figure is not a logarithmic scale, but a nominal scale. The average R_n values from glucose in the groups were 32, 28, 25, 19, and 16, and 36 was the greatest chain length found in this study.

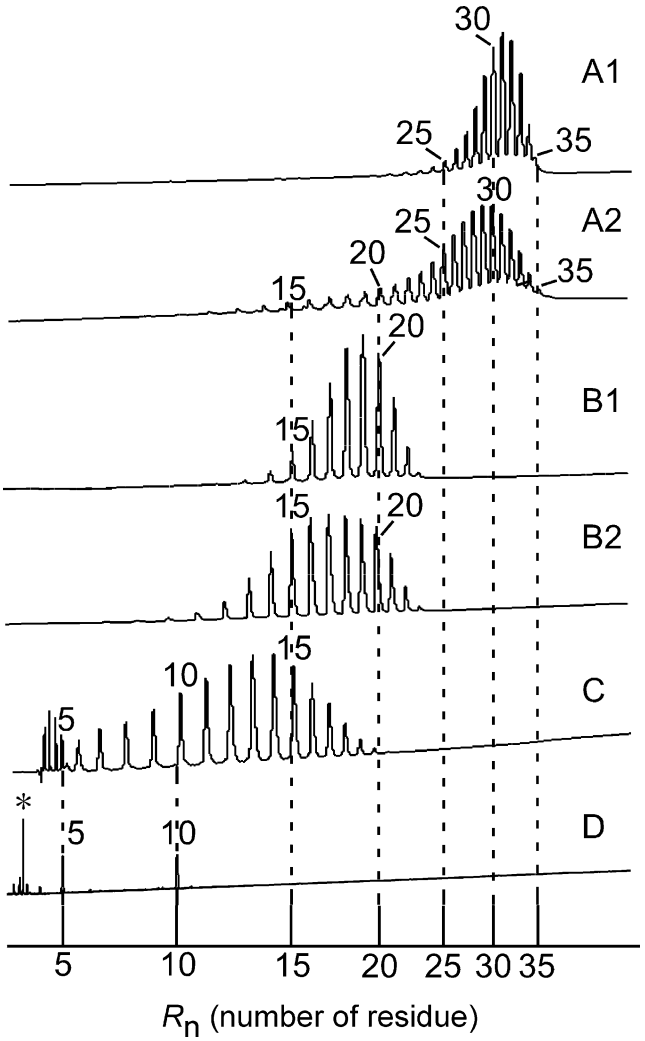

Fig. 1 Ion-pair chromatograms from the HPLC analysis of poly(ε-L-lysine) hydrochlorides (ε-PL·HCls) produced from 2% glucose (*A1* and *B1*) or 2% glycerol (*A2* and *B2*). The polymers were produced by the strains: USE-12 (*A1* and *A2*), USE-51 (*B1* and *B2*). Partially hydrolyzed ε-PL·HCl secreted by USE-82 (*C*). Chemically-synthesized ε-L-lysine oligomers·HCl consisting of five or ten residues (*D*). A total of 200 μg of each sample in Buffer A (see Materials and methods) was injected in (*A1*) to (*C*), and 30 μg in (*D*). *Asterisk* indicates impurity peaks

It is remarkable that most of the newly isolated strains produced a substantial amount of nearly monodispersed ε-PLs especially when glucose was used as a carbon source. One of the targets in polymer chemistry is to obtain a monodispersed polymer, which is critical for determining the relationship between the molecular weight and function.

All of the ε-PLs produced from 2% glycerol had a 10 to 20% lower M_n and a slightly broader M_w/M_n ratio than those synthesized from 2% glucose. Ion-pair HPLC analysis revealed that glycerol yielded ε-PLs with a lower R_n than glucose in the strains studied and that the greatest R_n produced from glucose was unchanged by the use of glycerol in most of the strains examined. USE-33 produced ε-PL with a rather broader M_w/M_n ratio from glucose than the rest of the strains and reduced the largest R_n of the polymer when glycerol was used (Table 1). Furthermore, glycerol reduced the molecular weight more (ca. 20%) in USE-33, USE-81, or USE-82 than in the rest of the strains (10 to 15%). The molecular weights of the polymers were not changed by culture time or culture medium compositions other than carbon source. These results indicate the molecular weight and polydispersity index of the ε-PLs were primarily determined by each producer strain.

NMR analysis of ε-PL from initially present 2% glycerol

It has been reported recently that ε-PL-glycerol ester accumulated in the culture medium when the polymer was produced from 5% glycerol with the addition of 1% glycerol every 24 h by NBRC 14147 (Nishikawa and Ogawa 2006). To examine the accumulation of such an ester under our culture conditions where initial carbon source level was 2% (the average level

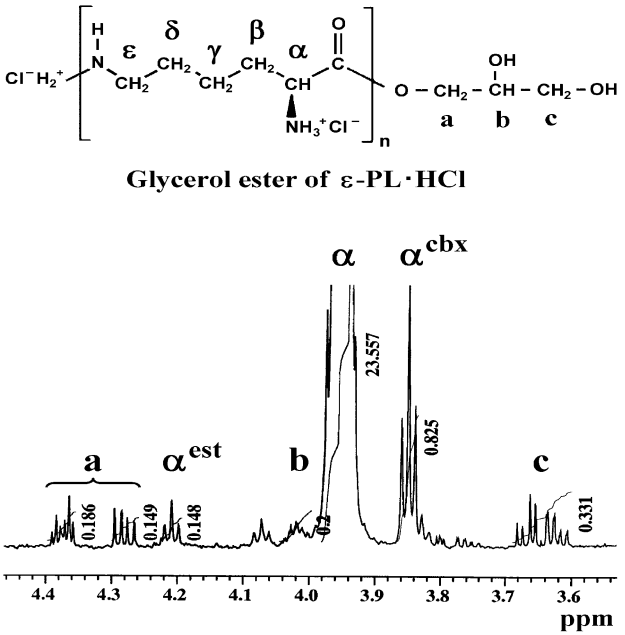

Fig. 2 Expanded regions of ^{1}H NMR spectrum of ε-PL·HCl produced from 2% glycerol by *S. albulus* NBRC 14147, containing the resonances for glycerol ester protons at C terminus, *a*, *b*, *c*, and ester α proton, and others. δ in ppm=3.62 and 3.66 (*c*), 3.7–3.8 (unidentified peaks), 3.83 and 4.07 (^{13}C satellite peaks of internal α), 3.85 (carboxyl terminus α, α^{cbx}), 3.95 (internal α, α), 4.02 (*b*), 4.21 (ester α proton, α^{est}), and 4.28 and 4.38 (*a*). The peaks at 4.38 may overlap with unidentified peaks. Numbers beside peaks indicate relative integrated areas of each peak to those of N terminus ε-protons being assumed to be 2. The resonances of *a*, *b* and *c* protons were assigned from cross-peaks to their bonding carbons at 69.95, 72.12, and 65.05 ppm, respectively, in the ^{1}H/^{13}C HMQC spectrum of the two-dimensional experiment, and those of α^{est} from the ^{1}H/^{13}C HMBC data, which showed cross-peaks to the neighboring three carbons in the esterified C terminus residue, i.e., β-, γ-, and carbonyl carbons at 32.3, 24.6, and 172.9 ppm, respectively. The full spectrum of ε-PL was given in Fig. 1 of Hirohara et al. (2006)

was ca. 1% during the polymer production), the purified ε-PL·HCls from glycerol by several strains including NBRC 14147 were subjected to 600 MHz NMR analysis. The signals of the glycerol ester were found in the polymers by some of the strains in both the expanded ^{1}H and ^{13}C NMR spectra, but no signals were observed by others in the either spectra. Figure 2 shows the expanded 600 MHz ^{1}H NMR spectrum of the ε-PL polymer by NBRC 14147 over the expected chemical shift range from 3.5 to 4.5 ppm (Ashby et al. 2005). It can be seen the resonances corresponding to the glycerol moiety of the ester and the esterified C terminus α-proton in addition to the internal and carboxyl terminus α-protons. The assignments of the glycerol moiety peaks were done from the $^{1}H/^{13}C$ HMQC data in the two-dimensional NMR experiments. The esterified α-proton was assigned from cross-peaks in the $^{1}H/^{13}C$ HMBC spectrum (see footnote of Fig. 2 for details). From the relative integrated areas of the esterified α-proton peaks to those of N terminus ε-protons at 3.01 ppm, the percentage of the ester at C terminus was estimated. The results were shown in the last column of Table 1. The esterification in NBRC 14147 was much lower under our culture conditions than that in the study of Nishikawa and Ogawa (2006). They observed nearly 100% of the esterification from 5% glycerol. Table 1 also tells termination through the esterification was dependent upon producer strain and that it preferentially occurred in the high molecular weight ε-PL producers. No signals were observed for polymers generated from glucose as anticipated.

Discussion

The relative height and the R_n of each peak, and hence the M_w/M_n values obtained seemed to be reliable since baseline separation was achieved in all of the cases with slight exceptions of USE-11 and USE-12 (see Fig. 1). The M_w/M_n of a polymer appeared to be determined possibly by the MALDI-TOFMS spectra as well. However, large nonquantitative effects were reported in the spectral intensities (Shimada et al. 2003). Hence, the ion-pair HPLC method was the most relevant to the estimation of the M_w/M_n of the ε-PLs in the present study.

Since the polymers with $R_n \geq 10$ showed optimum antimicrobial activity (Shima et al. 1984), the use of ε-PLs from USE-81 or USE-82 seems to be advantageous in terms of not having a bitter taste due to the addition of a large quantity of polymer. USE-82 may be especially attractive not only as a food preservative, but also for the exploration of ε-PL biosynthesis mechanism since this strain produced as a large amount of polymer as USE-11. The latter strain showed higher productivity than NBRC 14147 under our experimental conditions (Table 1). On the other hand, antiobesity action due to the inhibition of pancreatic lipase was reported to depend upon the number of amino groups in the molecule (Kido et al. 2003).

The elucidation of biosynthesis mechanism of ε-PL has been drawing extremely great attention in the study of naturally occurring poly(amino acid)s these days. Steady progress has been seen as a result of various attempts made in that direction. Recently, a gene encoding an ε-PL-degrading enzyme was cloned along with eight open reading frames in the flanking region surrounding the gene (Hamano et al. 2006). It was expected to identify ε-PL-synthesizing genes since both γ-PGA- and CGP-synthesizing genes were located close to the loci of their own depolymerase genes although ε-PL-synthesizing genes were not found in that study. The near monodispersity and relatively short chain length in this study may indicate that ε-PL was biosynthesized in a manner similar to the action of AMP-forming NRPSs between the two mechanisms described in the Introduction. In the model for this mechanism of polymerization, lysine monomers are first adenylated at their own carboxyl groups and then transferred into the active site sulfhydryl groups to form active aminoacyl thioester intermediates, which are characteristic of NRPSs (Marahiel et al. 1997; Finking and Marahiel 2004). The ε-PL synthetases catalyze repeated peptide bond formation reactions between the thio-esterified lysine and a nascent polylysine covalently bonded to a thiol template by incorporating lysine into the carboxyl terminus of the peptide. This model is consistent with the observations in a cell-free system (Kawai et al. 2003) and is compatible with the previous finding that SO_4^{2-} was essential for the ε-PL production (Hirohara et al. 2006). This prerequisite, despite of the presence of sulfate ion in the cell growth medium, might imply that each monomer forms an aminoacyl thioester substrate after the adenylation. However, we should stop speculating at this stage.

It was reported that in the microbial synthesis of poly (hydroxyalkanoates) (PHAs) from glycerol, the polymerization of P(3HB) was terminated through esterification by glycerol at the carboxyl terminus of the polymer, but that there was no evidence of a glycerol-derived chain termination in the synthesis of medium-chain-length PHA (mcl-PHA) with a repeat unit composition range from C4 to C14 (Taidi et al. 1994; Madden et al. 1999; Ashby et al. 2002, 2005). The termination model of PHA involves chain transfer in an acyl-enzyme intermediate being attacked at the active thioester site by a nucleophile such as water (Kawaguchi and Doi 1992), glycerol, 1,3-propanediol, and others (Madden et al. 1999). The reported results may indicate the chain termination is dependent upon acyl group in the thioester intermediate.

A similar termination model may be considered in the biosynthesis of ε-PL because of their similarity of active thioester intermediates during the polymerization. If this is the case, the dependence of transfer agent and its concentration in addition to producer strain may be seen in the termination of

ε-PL. Such dependence has been, in fact, observed in this study and by Nishikawa and Ogawa (2006). If two sorts of chain transfer agents were present in the production medium, they would compete with each other to terminate the polymerization. When 1% glycerol was present on the average, ca. 15% of the polymer molecules produced had glycerol ester terminus and more than 80% had free carboxyl one (Table 1). This indicated the polymerization was predominately terminated by water molecules in NBRC 14147. When 5% glycerol was maintained during the polymerization, on the other hand, Nishikawa and Ogawa (2006) showed almost of all the polymer molecules were terminated by glycerol in the same producer. All these results seem to support the termination model discussed here and hence are compatible with the elongation model involving the active thioester intermediates where the enzyme is acylated at an active sulfhydryl group(s) and lysine monomers are incorporated into the carboxyl terminus of ε-PL. However, at present, the mechanisms of elongation and termination of ε-PL are still unclear. More work needs to explain the biosynthesis mechanism of ε-PL.

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References

Ashby RD, Solaiman DKY, Foglia TA (2002) Poly(ethylene glycol)-mediated molar mass control of short-chain- and medium-chain-length poly(hydroxyalkanoates) from *Pseudomonas oleovorans*. Appl Microbiol Biotechnol 60:154–159

Ashby RD, Solaiman DKY, Foglia TA (2005) Synthesis of short-/medium-chain-length poly(hydroxyalkanoate) blends by mixed culture fermentation of glycerol. Biomacromolecules 6:2106–2112

Ashiuchi M, Misono H (2003) Poly-γ-glutamic acid. In: Fahnestock SR, Steinbüchel A (eds) Biopolymers, vol 7, Wiley, Weinheim, Germany, pp 123–173

Finking R, Marahiel MA (2004) Biosynthesis of nonribosomal peptides. Annu Rev Microbiol 58:453–488

Hamano Y, Yoshida T, Kito M, Nakamori S, Nagasawa T, Takagi H (2006) Biological function of the *pld* gene product that degrades ε-poly-L-lysine in *Streptomyces albulus*. Appl Microbiol Biotechnol 72:173–181

Hiraki J, Ichikawa T, Ninomiya S, Seki H, Uohama K, Seki H, Kimura S, Yanagimoto Y, Barnett JW Jr (2003) Use of ADME studies to confirm the safety of ε-polylysine as a preservative in food. Regul Toxicol Pharmacol 37:328–340

Hirayama C, Sakata M, Nakamura M, Ihara H, Kunitake M, Todokoro M (1999) Preparation of poly(ε-L-lysine) adsorbents and application to selective removal of lipopolysaccharides. J Chromatogr B 721:187–195

Hirohara H, Takehara M, Saimura M, Ikezaki A, Miyamoto M (2006) Biosynthesis of poly(ε-L-lysine)s in two newly isolated strains of *Streptomyces* sp. Appl Microbiol Biotechnol 73:321–331

Itzhaki RF (1972) Colorimetric method for estimating polylysine and polyarginine. Anal Biochem 50:569–574

Kawaguchi Y, Doi Y (1992) Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in *Alcaligenes eutrophus*. Macromolecules 25:2324–2329

Kawai T, Kubota T, Hiraki J, Izumi Y (2003) Biosynthesis of ε-poly-L-lysine in a cell-free system of *Streptomyces albulus*. Biochem Biophys Res Commun 311:635–640

Kido Y, Hiramoto S, Murao M, Horio Y, Miyazaki T, Kodama T, Nakabou, Y (2003) ε-polylysine inhibits pancreatic lipase activity and suppresses postprandial hypertriacylglyceridemia in rats. J Nutr 133:1887–1891

Kito M, Onji Y, Yoshida T, Nagasawa T (2002a) Occurrence of ε-poly-lysine-degrading enzyme in ε-poly-L-lysine-tolerant *Sphingobacterium multivorum* OJ10: purification and characterization. FEMS Microbiol Lett 207:147–151

Kito M, Takimoto R, Yoshida T, Nagasawa T (2002b) Purification and characterization of an ε-poly-L-lysine-degrading enzyme from an ε-poly-L-lysine producing strain of *Streptomyces albulus*. Arch Microbiol 178:325–330

Kito M, Takimoto R, Onji Y, Yoshida T, Nagasawa T (2003) Purification and characterization of an ε-poly-L-lysine-degrading enzyme from the ε-poly-L-lysine-tolerant *Chryseobacterium* sp. OJ7. J Biosci Bioeng 96:92–94

Lee H, Oyama K, Hiraki J, Hatakeyama M, Kurokawa Y, Morita H (1991) Microbial production and conformation of poly(ε-L-lysine). Chem Express 6:683–686

Madden LA, Anderson AJ, Shah DT, Asrar J (1999) Chain termination in polyhydroxyalkanoate synthesis: involvement of exogenous hydroxy-compounds as chain transfer agents. Int J Biol Macromol 25:43–53

Maeda-Yamamoto M, Mori Y, Osada K, Murakami H (1995) Enhancement of production of IgM and interferon-β in human cell lines by poly-lysine. Biosci Biotechnol Biochem 59:1842–1845

Marahiel MA, Stachelhaus T, Mootz HD (1997) Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem Rev 97:2651–2674

Nishikawa M, Ogawa K (2002) Distribution of microbes producing antimicrobial ε-poly-L-lysine polymers in soil microflora determined by a novel method. Appl Environ Microbiol 68:3575–3581

Nishikawa M, Ogawa K (2006) Inhibition of ε-poly-L-lysine biosynthesis in *Streptomycetaceae* bacteria by short-chain polyols. Appl Environ Microbiol 72:2306–2312

Oppermann-Sanio FB, Steinbüchel A (2003) Cyanophycin. In: Fahnestock SR, Steinbüchel A (eds) Biopolymers, vol 7, Wiley, Weinheim, Germany, pp 83–106

Shih I-L, Shen M-H, Van Y-T (2006) Microbial synthesis of poly(ε-lysine) and its various applications. Bioresour Technol 97:1148–1159

Shima S, Fukuhara Y, Sakai H (1982) Inactivation of bacteriophages by ε-poly-L-lysine produced by *Streptomyces*. Agric Biol Chem 46:1917–1919

Shima S, Matsuoka H, Iwamoto T, Sakai H (1984) Antimicrobial action of ε-poly-L-lysine. J Antibiot 37:1449–1455

Shimada K, Nagahata R, Kawabata S, Matsuyama S, Saito T, Kinugasa S (2003) Evaluation of the quantitativeness of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using an equimolar mixture of uniform poly(ethylene glycol) oligomers. J Mass Spectrom 38:948–954

Szókán Gy, Almás M, Krizsán K, Khlafulla AR, Tyihák E, Szende B (1997) Structure determination and synthesis of lysine isopeptides influencing on cell proliferation. Biopolymers 42:305–318

Taidi B, Anderson AJ, Dawes EA, Byrom D (1994) Effect of carbon source and concentration on the molecular mass of poly(3-hydroxybutyrate) produced by *Methylobacterium extorquens* and *Alcaligenes eutrophus.* Appl Microbiol Biotechnol 40:786–790

Tsujita T, Sumiyoshi M, Takaku T, Momsen WE, Lowe ME, Brockman HL (2003) Inhibition of lipases by ε-polylysine. J Lipid Res 44:2278–2286

Tsujita T, Takaichi H, Takaku T, Aoyama S, Hiraki J (2006) Antiobesity action of ε-polylysine, a potent inhibitor of pancreatic lipase. J Lipid Res 47:1852–1858

Yoshida T, Nagasawa T (2003) ε-Poly-L-lysine: microbial production, biodegradation and application potential. Appl Microbiol Biotechnol 62:21–26

Yoshida T, Hiraki J, Nagasawa T (2003) ε-Poly-L-lysine. In: Fahnestock SR, Steinbüchel A (eds) Biopolymers, vol 7, Wiley, Weinheim, Germany, pp 107–121