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Purification and characterization of poly-ε-lysine from *Streptomyces noursei* NRRL 5126

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The present article reports on the development of an effective downstream technique for the recovery of poly-ε-lysine (ε-PL), an unusual cationic homo-polyamide of L-lysine produced by fermentation using *Streptomyces noursei* NRRL 5126. Purification of ε-PL using cation exchange chromatography, ultrafiltration, solvent precipitation, and gel permeation chromatography was investigated. Loading of fermentation broth on Amberlite IRC 50 chromatographic column could purify the ε-PL with 95.84 % recovery. Subsequent fractionation with ultrafiltration (20kDa, and 5kDa) was found to be effective at minimal transmembrane pressure and optimal flux which resulted in 91.66 % product purity. Further, solvent precipitation and gel permeation chromatography which gave the highest possible purity (97.58%) and ε-PL yield (90.42%). Exclusion of ultrafiltration step negotiated the yield (88.62%) and purity (95.15) of ε-PL, if final application doesn't demand highly pure sample. Characterization with respect to molecular weight, thin layer chromatography, IR and NMR confirmed the identity of ε-PL in purified sample.

Keywords: Poly-ε-lysine, purification, ultrafiltration, gel filtration, characterization.

Introduction

Poly-ε-lysine (ε-PL), (S)-poly(imino(2-amino-1-oxo-1,6-hexanediyl)), is a naturally occurring homo-polymer of L-lysine with a degree of polymerization of 25–35 lysine units, and characterized by peptide bonds between the carboxyl and ε-amino groups of L-lysine[1]. It shows a wide antimicrobial spectrum against yeast, fungi, Gram-positive and Gram-negative bacteria species[1,2]. It has broad applications due to its notable biological activity, non-toxicity to mammals, water-solubility, and stability at high temperature. Recently its application as food preservatives, emulsifying agent, delivery agent, antiobese reagent, hydrogel, biodegradable material attracted attention of the researchers[3,4].

Different derivatives of ε-PL offer a wide range of unique applications such as highly water absorbable hydrogels, drug carriers, anticancer agent enhancer, and biochip coatings. ε-PL exhibit a variety of secondary structures such as a random coil, α-helix, or β-sheet conformations in aqueous solution. Moreover, transitions between conformations can be easily achieved using, salt concentration, alcohol content, pH or temperature as an environmental stimulus[1,5-8].

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In spite of commercial production and availability of ε-PL in the few countries, there are knowledge gaps still existing on many aspects of its production and downstream processing. The commercial importance of ε-PL demands not only a search for newer and better yielding microbial strains, but also economically viable bioprocesses for its large-scale production[9]. ε-PL is mainly produced by bacteria belonging to the family of Streptomycetaceae. It is currently produced industrially by *S. albulus*. Our previous work explicated the possibility to use *S. noursei* as a better alternative for *S. albulus* which is as yet not reported in the literature[10-12]. The cost of recovery of microbial polymers is a significant part of the total production cost of given product. Since *Streptomyces* sp. produces ε-PL as an extracellular biopolymer, its recovery and purification involves general downstream processing steps. The centrifugation/filtration followed by precipitation and different chromatographic separation techniques are the common steps involved in purification of ε-PL.

Kobayashi and Nishikawa[13] purified ε-PL from *Kitasatospora kifunense* by several purification steps. They adjusted the pH of culture filtrate to 7.0 and with 0.1 M HCl in ion exchange column. The eluate was lyophilized after precipitation and gel chromatography.Precipitation and ultrafiltration (UF) are the initial and most commonly used

concentration steps for volume reduction to aid further processing. Gel permeation chromatography (GPC) has been widely used for separation of biomolecules such as proteins, polysaccharides, and other high molecular weight substances and is mostly used as a final polishing step in purification protocol[14]. Literature on purification of ε-PL is in scarce and thorough knowledge about processing downstream is needed. The current study focuses on developing a purification process for ε-PL after production from *S. noursei* NRRL 5126.

Materials and methods

All the chemicals used in present study were of the AR grade and were purchased from Hi-Media Limited, Mumbai, India. Standard ε-PL was generously provided by Handary Bio-Engineering B.V. Netherlands. All the ion exchange and gel filtration resins were purchased from Amersham Biosciences and High Purity Laboratory Chemicals, India. Labscale tangential flow filtration system (Millipore) with hydrophilic BiomaxPolyethersulfone (MWCO 5kDa and 20kDa) membrane was purchased from Milipore, India. All chromatographic separations were performed using Biologic Duo Flow System from BioRad, California, USA.

Ion exchange chromatography

ε-PL was produced with *S. noursei* NRRL 5126 in 5L stirred tank bioreactor, BIOSTAT B plus (Sartorius, Germany) with 2 L working volume[12].After the completion of fermentation, the whole broth was centrifuged at 10000 g for 20 min to separate solid material and liquid. The supernatant was adjusted to pH 8.5 with 0.1N NaOH and filtered.The filtrate was applied on cation exchange resins (Amberlite IRC-50, Amberlite IRC 86, Amberlite IRC 748, Amberlite IRC120 -H and UNO sphere). Amberlite is a simple cation exchange resin showing high exchange capacity, is derived from carboxylic group. Acetic acid (0.2N) and water were used at a flow rate of 1ml/min, successively after sample load. Adsorption isotherm and breakthrough curve were fitted for exact resin load in column chromatography.

Determining the static binding capacity of the resin

The maximum binding capacity of the ε-PL on Amberlite IRC 50 was determined by adsorption studies in a batch mode. A series of centrifuge tubes containing 1ml Amberlite IRC 50 resin and different known concentrations of ε-PL with phosphate buffer (pH 7.0) were mixed with swirling on a rocker shaker for 2h. After 2h the supernatant was decanted from each tube and analyzed for ε-PL content.The amounts of ε-PL adsorbed (q) and un-adsorbed (c) were determined. A graph of equilibrium capacity (q*) Vs equilibrium concentration (c*) was plotted and maximum amount of ε-PL bound (concentration beyond which no further ε-PL was bound), q_{max} was determined.

$$q = \frac{q_{\max} c}{km + c} \quad \ldots (1)$$

km was determined from the straight-line plot of 1/q* against 1/c* from the slope (km/q_{max}) and the intercept ($1/q_{max}$). The Langmuir model was utilized to describe the equilibrium behavior in all the experiments.

Breakthrough curve analysis for dynamic binding capacity

The elution studies employing Amberlite IRC 50 column (3.6 cm) of 1.0 cm diameter was equilibrated with phosphate buffer pH 7.0 with a flow rate of 1 ml/min at room temperature (28±2 ºC). After equilibration, 1 ml sample was allowed to adsorb onto the resin in a packed bed column. Washing of the matrix with deionised water was done with two column volumes. Elution studies were carried out using 0.1N HCl. Fractions showing absorbance at 205 and 215nm were collected and analyzed for ε-PL content. The breakthrough curve was plotted from effluent ε-PL concentration Vs cumulative volume. Column performance was predicted by determining height equivalent to theoretical plate (HETP) value from the breakthrough data. Further studies were carried out by using different eluent flow rates (1,2, and 3ml/min) to analyze their efficacy to elute ε-PL. The eluate collected was neutralized with 0.1N NaOH to pH 6.5.

Cross flow filtration

Ultrafiltration (UF) was performed by using a lab scale tangential flow filtration system (Millipore) equipped with polyethersulfone flat membrane with molecular weight cut-offs of 5 and 20kDa. Millipore's Biomax® membrane comprising layers which are stacked together was continuously washed with water for 1h before it was used as UF medium.

In a tangential flow filtration (TFF) unit operation, filtrate flux increases with an increase in trans membrane pressure (TMP) up to a point and then it remains stable thereafter. The first part, where the

flux increases with the pressure, is a pressure dependent regime and the fouled membrane resistance is the primary limiting factor for it. Therefore, for a standard UF process, the optimum TMP, where nearly the highest flux is achieved without exerting excessive pressure or reaching exceedingly high protein wall concentrations is desirable[15].

The UF was conducted at room temperature (30±2 °C) and operating pressure was 0.3 to 2.0 bar. The sample after ion exchange elution was first fractionated with 20kDa(50 cm^2) membrane, which separated the sample into concentrate and filtrate solutions. When the volume of concentrate was reduced to 50 ml, the 100 ml deionized water was supplemented to continue the UF for 3 cycles. The filtrate obtained after first fraction was further fractionated with 5 kDa (50 cm^2) membrane and the operation was repeated as explained earlier. All the steps were culminated until the flux of the filtrate slowed down to 1.0 drop in 3.0 min. With this procedure, two concentrate solutions and one filtrate solution were obtained with different molecular weights of ε-PL. The volume and concentration of each fraction were assayed accurately to obtain the content of ε-PL in each fraction.

Parameters

TMP is the average applied pressure from the feed to the filtrate side of the membrane.

$$\text{TMP}=\left(\frac{P_f+P_r}{2}\right)-P_f \quad \ldots (2)$$

Apparent sieving (Sapp) is the fraction of a particular protein that passes through the membrane to thefiltrate stream based on the measurable protein concentrations in the feed and filtrate streams. A sievingcoefficient can be calculated for each compound in a feedstock.

$$S_{app}=\left(\frac{C_f}{C_b}\right) \quad \ldots (3)$$

Where C_f is concentration in filtrate and C_b is concentration in feed.

Filtrate flux (J_f) is the filtrate flow rate normalized for the area of membrane [m^2] through which it is passing.

$$J_f\left[\text{L/m}^2.\text{h}\right]=\frac{Q_f}{area} \quad \ldots (4)$$

Concentration factor (X) is the amount that the feed stream has been reduced in volume from the initial volume.

$$X=\frac{\text{Final product concentration}}{\text{Initial product concentration}}$$

The retentate which was obtained after UF (5 kDa) was further precipitated with methanol. This precipitate was filtrated and washed with ethanol. ε-PL thus obtained was further precipitated with the mixture of diethyl ether and ethanol (2:1) and different fractions were obtained.

Gel filtration chromatography

Gel filtration chromatography was used as a final polishing step in present purification scheme. Superdex peptide resin (recommended for molecular weight below 5000 D) was used in this study which is the first choice for high resolution, short run time and high recovery. 500 mg sample after solvent precipitation was loaded with 1ml/min flow rate. A bed height of 7.0 cm with total column volume12 ml was used for final polishing step. Absorbance was measured at 205, 215, 265, and 280 nm.

After analyzing the purification protocol for ε-PL by *S. noursei* NRRL 5126, it was presumed that, some downstream processing steps can be minimized on the basis of product yield, percent purity and product loss at each step. UF, although recommended by few researchers[16-18], was not found to be a mandatory step to be included in the final purification protocol. Hence, the purification protocol after cation exchange chromatography was repeated as explained earlier by excluding UF, to check its efficacy both in terms of percent purity and ε-PL yield. The results excluding UF step were compared with results including UF step during the purification protocol.

Characterization of ε-PL

After elution of ε-PL from gel filtration chromatography the eluate was neutralized with 0.1 M NaOH to pH 6.5 if required. Sample was dried with lab scale freeze drier (Labconco, USA) and further characterized for its authentication. Molecular weight and polydispersity of ε-PL produced by *S. noursei* NRRL 5126 was measured by gel permeation column. HPLC system (Agilent) fitted with TSK gel permeation column and refractive index (RI) detector was used to detect standard as well as sample ε-PL. Although the standard molecular

weight markers are not available in the market for ε-PL, different general polymeric markers (molecular weight 10,000 to 723,500 Da) were used to construct a standard curve to estimate the molecular weight. Column was developed with 0.1 M phosphoric acid at a flow rate of 0.4 ml/min.

Amino acid analysis was performed using thin layer chromatography (TLC). The purified ε-PL was hydrolyzed with 6N HCl at 160 °C for 24h and used for amino acid analysis. TLC was performed on a cellulose plate with solvent systems of butanol–acetic acid–water (3:1:1, v/v) and ethanol–water (63:37, v/v). Amino acids (lysine) were detected after 0.2% ninhydrin spray with ferric reagent[19]. Purified ε-PL sample was characterized by IR for its structural elucidation. The H^1- and C^{13}-NMR spectra were recorded and the chemical shift of ε-PL was also analyzed.

Analysis of ε-PL

The ε-PL concentration was measured using the method of Shen*et al*.[20]. The method is based on selective binding of trypan blue by ε-PL. Briefly, 1 ml of supernatant was incubated with 0.1 mM phosphate buffer and 120 µl of trypan blue solution (1 mg/ml), thoroughly mixed and incubated at 37°C. Absorbance was measured after centrifugation at 580 nm on Heliose (α) UV-VIS spectrophotometer (Thermo Electron, Germany). Percent purity of ε-PL was monitored by high performance liquid chromatography (HPLC) assay method, previously reported by Kahar *et al*.[21]. A TSK gel ODS-120T column (Tosho Co. Ltd., Tokyo, 4.6 mm × 250 mm, 10 mm) was used for ε-PL detection. The sample was eluted with 0.1% H_3PO_4 at a flow rate of 0.4 ml/min and chromatograms were analyzed at 205,215,265 and 280nm.

Results and discussion

The strain *S. noursei* NRRL 5126 used in the present study could produce ε-PL upto 2 g/l with optimized lab scale fermenter conditions[12]. Fermentation broth was first centrifuged to remove cells at 10,000 g for 15 min. The pH of the fermentation broth was adjusted to 8.5 with 1N NaOH and supernatant containing ε-PL was then analyzed after subsequent purification steps.

Ion exchange chromatography

Among the cation exchange resins screened and adsorption isotherm analyzedfor higher ε-PL binding with effective elution pattern, Amberlite IRC 50 was found to be best suited resin (Table 1). The % ε-PL binding was highest on Amberlite IRC 50 while lowest on Amberlite IRC 120H. The higher ion exchange capacity of Amberlite IRC 50 and Amberlite IRC 86 is derived from the carboxylic acid group present on the resin. Contrary to this Amberlite IRC 120H consisted sulphonated polystyrene group while Amberlite IRC 748 carries iminodiacetic acid functional group. Less than 11% reversible swelling capacity of Amberlite IRC 120H for H^+ and higher selectivity of Amberlite IRC 748 for heavy metals made them unsuitable for ε-PL separation. ε-PL did not bind to UNO sphere as expected which is known to bind the proteins.

The static binding capacity of the resin

Equilibrium adsorption isotherm was studied to find out adsorption isotherm pattern for purification of ε-PL using Amberlite IRC 50. The adsorption isotherm data was used to determine the maximum adsorption capacity of the matrix (q_{max}) and Langmuir constant (km) value. km is the affinity of ε-PL for the adsorbent matrix. km depends on the strength of a binding forces on the surface and its value is always greater than zero. The ε-PL adsorption followed a typical Langmuir type of isotherm for Amberlite IRC 50. Adsorption of ε-PL onto Amberlite IRC 50 increased to a certain point (q_{max}), beyond which no further adsorption of ε-PL was observed. The maximum capacity of the resin (q_{max}) for ε-PL was found to be 1.17mg/ml of resin. A plot of 1/q Vs 1/c gave a linear correlation with regression coefficient of 0.90, confirming the adsorption to be of the Langmuir type (Fig. 1).The value for km was found to be 0.46mg/ml for Amberlite IRC 50.

Column studies on elution conditions of ε-PL

Isocratic elution pattern was used with 0.1N HCl for effective purification and it was further analyzed

Table 1—Screening of cation exchange resins for ε-PL binding and elution

Resin used	Amberlite IRC 50	Amberlite IRC 86	Amberlite IRC 748	Amberlite IRC 120H	UNO sphere
Sample loaded (g/l)	1.85	1.85	1.85	1.85	1.85
Elution (g/l)	1.81±0.03	1.57±0.03	1.75±0.02	0.52±0.03	-
Binding (%)	97.84	84.86	72.97	28.11	0

at four wavelengths as reported in materials and method section. The breakthrough curve experiments for Amberlite IRC 50 resulted in total binding capacity of 102 µg ε-PL/ml of resin. A resin bed height of 2.4 cm with flow rate of 2 ml/min was found to be optimum and resulted in 95.56% ε-PL binding.This cation exchange column resulted in 92.36% purity with 1.67% loss of ε-PL (Table 2). Because of the selectivity of resin for hydrogen ion, any adsorbed cation can be desorbed easily with regeneration efficiency by treatment with dilute mineral acids such as HCl.

Ultrafiltration

The eluent from ion exchange chromatography was further used for UF. The solution was firstly fractionated with 20kDa and filtrate was further processed with 5kDa membrane. A distinct feature of UF is the tendency of biomolecules to foul the membrane by adsorption or causing loss of flux[22]. The flux can be set by controlling the TMP and flow velocity at which a cross flow filtration unit is being operated. High wall shear stress was observed at the membrane surface with high flow rate which resulted in decrease in the membrane fouling.

Table 3 detailed the effect of TMP, filtration flux, and ε-PL yield after 5kDa membrane filtration. Increasing the TMP from 0.034 to 1.72 bar increased the permeate flux linearly from 48 to 120 l/m^2.h This was in accordance with the thin film law of Blatt *et al*.[23] where the permeate flux increases significantly with velocity, and remains stable at high levels of TMP[23,24]. Neither ε-PL yield, nor its purity was significantly affected with higher TMP. Hence, 0.34 and 0.69 bar feed pressure and retentate pressure, respectively, were selected as optimal. The percent purity after UF was found to be 93.14% (Table 2).

UF in present study was expected to concentrate and increase the purity of ε-PL before it can be applied for solvent precipitation. Very small increase (0.8%) in purity with lower dilution factor (0.29 g/l) was observed after UF. These results encouraged us to carry out the remaining purification protocol by excluding UF step.

Precipitation, initially with methanol, followed by mixture of diethyl ether and ethanol was used further for concentration of ε-PL. Methanol alone could precipitate and concentrate ε-PL upto 3.0643 mg/ml while mixture of ethanol and diethyl ether concentrated it upto 3.039 mg/ml. Insignificant loss of 0.55% with 93.68% purity was observed after solvent precipitation with highest concentration factor of 6.61 g/l. The percent yield and purity after precipitation was also not significantly high when compared with ion exchange chromatography (Table 2). Highest concentration factor of this step makes it suitable to be used prior to gel chromatography. Concentrated sample (3.039 mg/ml) was useful during sample loading and peak resolutions, and hence solvent precipitation was retained in the purification protocol of ε-PL although it did not increase the sample purity and yield.

Gel filtration chromatography

The final polishing step with gel filtration chromatography (Superdex column, 12 ml) was used to fractionate ε-PL. Two protocols namely, protocol-A with UF, and protocol-B without UF were used for final polishing step. Protocol-A with UF step showed two fractions with retention volumes of 5.0 ml and 9.0 ml, of which the first fraction (smaller) did not show ε-PL activity, and hence neglected for further calculations. Second fraction (larger) was found to exhibit the ε-PL activity with only 1.5% polymer loss (Table 2).The other gel filtration

Table 2—Overall purification of ε-PL from *S. noursei* NRRL 5126 using different chromatographic and concentration techniques

Purification step	End volume (ml)	ε-PL (mg/ml)	% Purity	% Yield[a]	Concentration factor (mg/ml)
Fermentation broth	5	2	-	100.00	1.0
Cation exchange chromatography	6	1.60	92.36	95.84±0.68	0.8
Ultrafiltration	20	0.46	93.14	91.66±0.53	0.29
Solvent precipitation	3	3.04	93.68	91.17±0.59	6.61
GPC with UF*	5	90.42	97.58	90.42±0.71	0.90
GPC without UF*	5	88.62	95.15	88.62±0.57	0.88

[a]Calculated with respect to the content in the fermentation broth

All the purification steps were performed sequentially except gel filtration chromatography

*100 mg/ml sample (after precipitation) was loaded

parameters are detailed in Table 4. With this method, 97.58% pure ε-PL was achieved with only 1.5% polymer loss.

Second protocol-B without UF, was carried out similarly as like protocol-A, excluding UF step from it. The ε-PL was eluted after 9.2 ml retention with prior small peak after 5.0 ml as a contaminant. Protocol-B showed lesser ε-PL yield (88.62%) and percent purity (95.15%) as compared to protocol-A (Table 4).

By considering the process efficiency, percent purity and application area, the selection of downstream process for ε-PL with *S. noursei* NRRL 5126 can be explicated. Applications such as ε-PL as a drug carrier, hydrogel formation, as a coating material can be explored with the purification protocol-B wherein UF step is less important and can be eliminated to reduce the process cost, while compromising with the yields and purity. Conversely, ε-PL application in gene delivery system, nanoparticle formation and interferon inducers needs highest purity, and hence the protocol-A, including UF step is desirable.

Characterization of purified ε-PL from *S. noursei* NRRL 5126

The purified ε-PL was characterized for product homogeneity and structure elucidation by IR, proton (H^1)-NMR, carbon (C^{13})-NMR and molecular weight measurement.The IR spectra of purified ε-PL from *S. noursei* NRRL 5126 explicited N-H, C=O and C-H stretching at desired wavelengths. The determination of ε-PL of definitive molecular size is difficult, because authentic standard polymers are not available commercially.The molecular weight of ε-PL obtained from *S. noursei* NRRL 5126 was found to be below 10 kDa. The lowest possible molecular weight cut off marker that we could reach was 10 kDa, and hence presuming the molecular weight to be below 10 kDa based on retention time and standard curve observed with HPLC-RI detector. It is known that the molecular weight of ε-PL varies from 3 to 6kDa depending upon the number of lysine monomers in the chain[8,18]. Under the conditions used in this study, the molecular weight of ε-PL is comparable to the results obtained so far with other species.

The 6N HCL hydrolysate of the purified material obtained from *S. noursei* NRRL 5126 solely consisted of lysine. TLC of the hydrolysate performed on a cellulose thin-layer plate and visualized with 0.2% ninhydrin ferric reagent indicated a single spot with R_f value identical to that of authentic lysine. The NMR of purified sample of ε-PL also matched well with standard ε-PL and peaks corresponding

Table 3—Parameters affecting ultrafiltration efficacy during ε-PL purification produced by *S. noursei* NRRL 5126

Feed pressure (bar)	Retentate pressure (bar)	TMP (bar)	% Yield[a]	Avg. permeate flux[a] ($l/m^2.h$)
0.34	0.69	0.075	98.65±0.75	48±0.65
0.69	0.89	0.100	98.96±0.98	72±0.85
1.39	1.58	0.095	98.16±0.89	96±0.39
1.72	2.1	0.190	97.65±0.96	120±0.96

[a]Results are mean±SD of three determinations

Table 4—Gel filtration chromatography parameters during final polishing step of purification for ε-PL from *S. noursei* NRRL 5126

Parameter	Values	
	With UF	Without UF
Sample loaded (mg)	500	500
Column volume (Vt) (ml)	12	12
Void volume (Vo)(ml)	5.0	5.0
Exclusion volume (Ve) (ml)	9.0	9.2
Stationary phase volume (Vs) (ml)	7.0	7.0
Distribution coefficient average K_{av} =(Ve-Vo)/ (Vt-Vo)	0.57	0.6
K_d – (Ve-Vo)/Vi	1.33	1.50
Number of theoretical plates (N)	183	1.69
HETP (mm)	0.37	0.35
Yield (%)	90.42	88.62
Purity (%)	97.58	95.15

to the expected functional groups. Since, ε-PL is a homopolymer containing a peptide linkage between the α-carboxyl group and the ε-amino group of $_{L}$-lysine, the environment of ε-methylene at N-terminal and α-methine at C-terminal are different from those in the intermediate part of $_{L}$-lysine units. These differences can be used to determine the degrees of polymerization and the number the average molecular weights of ε-PL. Jia *et al*.[18] have measured the ^{1}H NMR spectra of ε-PL with different molecular weight cut-offs. Chemical shift of ε-PL in various solvents have been reported and studied by various researchers[3,8,18].They concluded that the chemical shifts of corresponding hydrogen in ε-PL were all in very similar regions, reflecting similar conformations of ε-PL with different molecular weights in aqueous solution.

Conclusions

ε-PLsecreted by *S. noursei* NRRL 5126 was successfully purified and fractionated with various separation and concentration techniques. Cation exchange chromatography and gel filtration were found to be simple and effective techniques to increase the ε-PL purity with higher yields. Minimal TMP and optimal flux resulted in 91.66% ε-PL yield with 93.14% purity. UF step can be eliminated from the purification protocol if the final product application does not demand highest purity of ε-PL. Gel filtration chromatography with and without UF was found to be suitable polishing step to increase the purity of sample with no/insignificant product loss. IR, NMR and TLC confirmed the presence of ε-PL in purified sample, while HPLC-molecular weight determination and TLC identified its exclusive existence. Overall purification after gel filtration reached to 97.58% pure ε-PL with 90.42% sample yield.

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