Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Preparation, characterization and antimicrobial activity of ε-poly-L-lysine with short chain length produced from glycerol by *Streptomyces albulus*

Xu-Sheng Chen*, Kai-Fang Wang, Gen-Cheng Zheng, Yang Gao, Zhong-Gui Mao*

The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

ARTICLE INFO

Keywords:
ε-Poly-L-lysine
Antimicrobial agent
Chain length control
Glycerol
Food preservative

ABSTRACT

ε-Poly-L-lysine (ε-PL) serves as a biological preservative in food industry for many years in several countries. However, the naturally occurring ε-PL with the chain length of 25–35 L-lysine residuals exhibits bitter taste. Thus, the decrease of chain length at an appropriate range has become very critical for the wide application of ε-PL. Herein, we proposed an efficient strategy for the short chain ε-PL production with the high yield (39.84 g/L), high purity (98.81%) and high recovery ratio (72.59%) by fed-batch fermentation using glycerol as carbon source. The short chain ε-PL with 8–32 L-lysine residuals showed different secondary structures and better antimicrobial activity towards yeast than naturally occurring one. As a result, a simple, low-cost and safe strategy for high-level production of short chain ε-PL was developed, which may enlarge the application of ε-PL in the food industry.

1. Introduction

ε-Poly-L-lysine (ε-PL) is a unique homo-poly(amino acid), produced by various *Streptomycetaceae* bacteria and some *Bacillus* as well as a few filamentous fungi as a secondary metabolite [1–3]. The typical ε-PL is generally composed of 25–35 L-lysine residuals linked by ε-amino and α-carboxyl groups with a molecular weight range from 3200 to 4500 Da. It has a wide antimicrobial spectrum of microorganisms, including Gram-positive and Gram-negative bacteria, yeast and fungi, which is mainly attributed to its isopeptide bond and multi-amino groups [4]. Notably, ε-PL strongly inhibits many foodborne pathogens such as *Escherichia coli* O157:H7 [5], *Listeria monocytogenes* [6], *Staphylococcus aureus* [7]. Moreover, the compound is biodegradable, water-soluble, thermal-stable and non-toxic [8]. Therefore, the ε-PL is identified as a generally recognized as safe (GRAS) for use in food industry as an antimicrobial agent by the US Food and Drug Administration [9]. Currently, it is allowed to be used in Japan (1980s), South Korea (1990s), United States (2004), and China (2014) as a biological preservative in food industry [10].

As an emerging food preservative, ε-PL exhibits more advantages than conventional preservatives. Compared with chemically synthesized preservatives, such as potassium sorbate, sodium benzoate and nitrite, ε-PL shows high safety and environmental friendly properties. For these plant or animal tissue derived preservatives, such as pectin, protamine and propolis, ε-PL shows low cost and high stability. For the same origin of microbial preservatives, such as nisin and natamycin, ε-PL has a wider usage pH ranges and high water solubility as well as broad antimicrobial spectrum. Thus, ε-PL is an ideal food preservative with more advantages than traditional ones.

Actually, the antimicrobial activity of ε-PL is primarily depended on its chain length. It is reported that ε-PL with less than 9 L-lysine residues is known to have no antimicrobial activity [11]. However, naturally occurring ε-PL with 25–35 L-lysine residues exhibits unpleasant bitter taste, which would limit its application in food industry [12]. Thus, the controlling of ε-PL at an appropriate chain length has an important significance for its wide application. At present, four strategies have been employed to produce the short chain ε-PL. Since each ε-PL-producing strain produces ε-PL with different chain length, such as *S. albulus* NBRC14147 with 3.2–4.5 kDa [2]; *Streptomyces* sp. USE-51 with 2.3 kDa [13]; *Kitasatospora* sp. PL6-3 with 5 kDa [14], it is ideal to directly screen strain with short chain ε-PL producing from environment. However, the high-yield production of ε-PL consisting of 5–20 L-lysine residues only reached 4.5 g/L [15], indicating it has a long way to use this strain in industry. The second strategy is addition of aliphatic short chain polyols or sulfated β-cyclodextrin into culture during fermentation. It was found pentamethylene glycol could effectively regulate the chain length of ε-PL [16], and polyanionic sulfated β-cyclodextrin was successfully reduced the molecular weight of ε-PL from 3.5 to 4.5 kDa (27–35 mer) to less than 2.5 kDa (~19 mer) [17]. These satisfactory results represented the strategies are promising, but the majority of multi-hydroxyl compounds are difficultly applied in industry in terms of food safety. In respect to the third strategy, the Hamano group

* Corresponding authors.
E-mail addresses: chenxs@jiangnan.edu.cn (X.-S. Chen), maozg@jiangnan.edu.cn (Z.-G. Mao).

https://doi.org/10.1016/j.procbio.2018.03.001
Received 2 December 2017; Received in revised form 13 February 2018; Accepted 1 March 2018

proposed the shortening of ε-PL chain length by ε-PL-synthetase engineering through linker regions mutagenesis [18]. Unfortunately, the short chain ε-PL production of variants is too low and the mechanism is not fully understood. In the previous study, we have prepared the short chain ε-PL from the naturally occurring ε-PL by the ε-PL-degrading enzyme, which is an endo-type protease and purified from *S. albulus* M-Z18 [19]. Although this method has successfully achieved the preparation of short chain ε-PL as the fourth strategy, it is difficult to ensure the consistency of chain length distribution among different batches. Moreover, the cost of enzyme preparation is very high and inefficient. Therefore, there is still a lack of effective method to prepare ε-PL with short chain length, especially in a large amount.

It was reported short-chain aliphatic polyols could inhibit L-lysine polymerization via esterification and produce short chain ε-PL. However, glycerol, one of polyols with high food safety and low cost, was approved non-efficient in the production of ε-PL with short chain length [16]. In this study, we carried out short chain ε-PL preparation in large amount from glycerol by *S. albulus* M-Z18, and the characteristics of chain length distribution, secondary structure and antimicrobial activity of the short chain ε-PL were also investigated.

2. Materials and methods

2.1. Strain and medium

Streptomyces albulus M-Z18 was used for ε-PL production and preserved in our lab. The spores of *S. albulus* M-Z18 were preparation and maintained on the modified Bennett's agar slant, which contained (per liter): glucose, 10 g; beef extract, 1 g; polypepton, 2 g; yeast extract, 1 g; and agar, 18 g, the pH adjusted to 7.0 with 2 M NaOH before sterilization. The M3G medium was used for seed cultivation, which composed of (per liter): glucose, 50 g; yeast extract, 5 g; $(NH_4)_2SO_4$, 10 g; $K_2HPO_4{\cdot}3H_2O$, 0.8 g; KH_2PO_4, 1.36 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; $ZnSO_4{\cdot}7H_2O$, 0.04 g; $FeSO_4{\cdot}7H_2O$, 0.03 g, the pH adjusted to 6.8 with 2 M NaOH solution before sterilization. Fermentation medium was developed in our previous study [20] and made some modification used for ε-PL production, which contained (per liter): glycerol/glucose 60 g, $(NH_4)_2SO_4$ 8 g, yeast extract 10 g, KH_2PO_4 5 g, $MgSO_4{\cdot}7H_2O$ 2 g, $FeSO_4{\cdot}7H_2O$ 0.1 g, the pH adjusted to 6.8 with NH_4OH solution (24%, w/v) after sterilization.

Escherichia coli CICIM B0013, *Bacillus subtilis* CICIM B0629, *Micrococcus tetragenus* CICIM B0126, *Staphylococcus cremoris* CICIM B1294, *Saccharomyces cerevisiae* CICIM Y0086, *Pichia pastoris* CICIM Y0154, *Torulopsis globosa* CICIM Y1017, *Aspergillus niger* CIFIM F0015 *Aspergillus oryzae* CICIM F1005, *Penicillium chrysogenum* CICIM F0058 were used for antimicrobial test and gifted from China University Industrial Microbial Resources and Information Center (CICIM), Jiangnan University. The LB medium was used for the growth of *E. coli* CICIM B0013, *B. subtilis* CICIM B0629, *M. tetragenus* CICIM B0126, *S. cremoris* CICIM B1294, which contained (per liter): NaCl, 10 g; tryptone, 10 g; yeast extract, 5 g; the pH adjusted to 7.2-7.4 with 2 M NaOH before sterilization. YPD medium was used for the growth of *S. cerevisiae* CICIM Y0086, *P. pastoris* CICIM Y0154, *Torulopsis globosa* CICIM Y1017, which contained (per liter): yeast extract 10 g; peptone 20 g; glucose 20 g. The PDA medium was used for the growth of *A. niger* CIFIM F0015, *Aspergillus oryzae* CICIM F1005, *Penicillium chrysogenum* CICIM F0058, which contained (per liter): glucose, 20 g; potato juice, 200 g; natural pH.

All the media were sterilized in an autoclave at 121 °C for 20 min. In addition, glucose was autoclaved separately and mixed together prior to use in each case. All chemicals, except organic nitrogen source (beef extract, yeast extract, polypepton, tryptone, peptone are in biochemical grade), were used in analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of ε-PL-contained fermentation broth

In our previous study, a pH control strategy, pH shock, was developed in fed-batch fermentation for the overproduction of ε-PL by *S. albulus* M-Z18 [21]. Though a high production of ε-PL was obtained (54.7 g/L), a large amount of biomass (76.35 g/L) and a low yield (8.6%) hurt its economy. It would also not be beneficial to separation and purification of the ε-PL from that culture broth. To reduce the high biomass, sole organic nitrogen source (yeast extract) was used to replace of the mixed organic nitrogen source (fish meal and corn syrup) as shown in the above medium component, and other conditions were as following:

Aliquot 0.1 mL spore suspension (2×10^5 spores/mL) was inoculated into a 500 mL flask containing 80 mL M3G medium and incubated at 30 °C on a rotary shaker with 200 rpm for 24 h. After the adjustment of temperature, aeration rate, agitation speed and initial pH at 30 °C, 0.5 vvm, 200 rpm, 6.80, respectively, this culture with approximately 300 mL volume was transferred as seed into a 5-L fermenter (Biotech-5BG, Baoxing Bio-Engineering Equipment Co., Ltd., Shanghai, China) contained 3.2 L fermentation medium. In the process of fermentation, the pH shock strategy was adopted: when the pH was naturally declined from the initial 6.80–5.00, maintained pH 5.00 for 10 h by automatic addition NH_4OH (12.5%, w/v), then allowed pH dropping naturally for 12 h, finally restoring pH to 4.00 and kept it constant until the end of fermentation. Dissolved oxygen (DO) was set above 30% of air saturation before pH declined to 4.00 and above 20% of air saturation afterwards, which was controlled by adjusting agitation during fermentation before the agitation reaching the maximum speed. When agitation reached to 800 rpm, aeration rate was then manually increased in the stepwise of 0.5 vvm with a range of 0.5-2.0 vvm. During the fermentation, pH and DO were monitored online by pH and DO electrodes, respectively (K8S-225 and InPro6800, Mettler Toledo, Greifensee, Switzerland). When the concentration of glucose or glycerol was low than 10 g/L, sterilized glucose (800 g/L) or glycerol (1200 g/L) solution were automatically added by peristaltic pump to maintain their concentrations at about 10 g/L. When residual ammonia nitrogen (NH_4^+-N) was low than 0.5 g/L, sterilized $(NH_4)_2SO_4$ solution (600 g/L) was fed and maintained its concentration until the end of fermentation.

After 192 h fed-batch fermentation, about 5.0 L fermentation broth was harvested, which mainly contained (g/L): ε-PL, ~39; mycelia (dry cell weight), ~50; inorganic ions (Na^+, NH_4^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- 1.15, SO_4^{2-}), ~14; amino acids, ~1; protein, ~2; organic acids, ~3; residual carbon source, ~5; and etc.

2.3. Separation and purification of ε-PL from fermentation broth

In our previous work, a successive process for separation and purification of ε-PL from fermentation broth including flocculation, filtration, ultrafiltration, ion-exchange chromatography, and decoloration was developed [22]. Finally, the purity and recovery of ε-PL reached 90.2% and 75.0%, respectively. Although the purity of ε-PL had enhanced by 4.88-fold than the original, it was also below the standard requirement (> 95%). To further improve the purity of ε-PL, the developed process was modified as following: (1) flocculation and filtration operations were replaced by dilution and centrifugation for the removal of high biomass from fermentation broth in order to reduce the hydrochloric acid addition and decrease the subsequent desalination operation load; (2) active carbon was replaced of macroporous resin for decoloration to simplified operation. The detailed process of ε-PL separation and purification from fermentation broth was as following:

The 1.0 L fermentation broth with about 30% (v/v) wet mycelia was centrifuged at 5000*g* for 15 min, and repeatedly washed precipitate 3 times with 500 mL deionized water in each. A total 2.0 L of cell-free supernatant was withdrawn for the ultrafiltration with the cutoff molecular weight of 30 kDa membrane (0.1 m^2) using a Pellicon 2 mini