Biochemical Engineering Journal 42 (2008) 270–275

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

Enhancement of poly(arginyl-histidine) production by *Verticillium kibiense* E18

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ARTICLE INFO

Article history:
Received 25 December 2007
Received in revised form 15 July 2008
Accepted 18 July 2008

Keywords:
Poly(arginyl-histidine)
Culture conditions
Verticillium kibiense
Cationic peptide
Antimicrobial peptide
Poly(amino acid)

ABSTRACT

An ergot fungus *Verticillium kibiense* E18 produced two cationic peptides, ε-poly-L-lysine (ePL) and poly(L-arginyl-D-histidine) (PRH). The ePL was used as a food preservative, and it was expected that PRH would be used as a novel material, such as cationic and antimicrobial peptide. To enhance PRH production of strain E18, various culture conditions were investigated. Glucose was a suitable carbon source for PRH production, although glycerol was a suitable carbon source for growth. The cultivation temperature significantly influenced both cell growth and PRH production. The optimal temperatures for cell growth and PRH production were 28 and 30 °C, respectively. Moreover, strain E18 produced more PRH when an additional 5.0 μg/L $FeSO_4 \cdot 7H_2O$ was added to the production medium. Under optimal conditions, strain E18 enhanced PRH production, while suppressing ePL production. The maximum PRH production was 183.9 mg/L, which is approximately 60-fold higher than that of the initial culture condition.

1. Introduction

Poly(amino acid)s are useful materials for antimicrobial agents and biodegradable polymers. Various poly(amino acid)s have been chemically synthesized and investigated for a wide variety of technical applications. On the other hand, some microorganisms produce poly(amino acid)s such as ε-poly-L-lysine (ePL) [1,2], poly(γ-glutamic acid) (PGA) [3], multi-L-arginyl-poly-L-aspartic acid (cyanophycin) [4,5], and poly(γ-glutamyl-cysteinyl)glycine (phytochelatin) [6]. Recently, it has been reported that an ergot fungus *Verticillium kibiense* (formerly *Epichloë kibiensis*) E18, that was newly isolated as an ePL producer, also produces poly(arginyl-histidine) (PRH) as the fifth biosynthesized poly(amino acid) [7]. The PRH was a D,L-α-peptide that consists of repeating dipeptide units composed of alternating residues of an L-arginine and D-histidine linked α-peptide bond (Fig. 1) and that shows antimicrobial activity [8,9]. PRH has the unique feature of dipeptide repetition with an α-peptide bond, while other poly(amino acid)s have special structural linkages involving ε-amino and γ- and β-carboxylic groups.

Among these biosynthesized poly(amino acid)s, ePL has been used as a food preservative, and there have been expectations that PGA would be used as a material for biodegradable polymer. PRH is a histidine- and arginine-rich peptide containing each 5 residues of histidine and arginine. As to an arginine-rich peptide, HIV-1 Tat protein is well known. HIV-1 Tat protein has been successfully used for the efficient intracellular delivery of molecules including proteins, oligonucleic acids, and liposomes. Many arginine-rich peptides such as HIV-1 Rev and octaarginine are efficient intracellular deliverers of these molecules [10]. Not only the linear peptides but also the branched-chain peptides showed efficient internalization with an optimal number of arginines (approximately 8 residues). As to a histidine rich peptide, histatins 1, 3, and 5 from human parotid secretion are well known. Because of their potent in vitro antifungal activity and non-toxicity to humans, salivary histatins have therapeutic potential as drugs against oral candidiasis. Histatins 1, 3, and 5 contain 28, 32, and 24 amino acid residues, respectively, and also contain 7 histidine residues [11,12].

Given this background, PRH, which shows antimicrobial activity and is an arginine- and histidine-rich peptide, has the potential for the use as a functional material for gene delivery or an antimicrobial agent. However, PRH productivity of *V. kibiense* E18 has been quite low (approximately 1–5 mg/L), not enough to provide for the development of an application. Recently, microbial activity has been improved by genetic engineering. However, the biosynthesis mechanism by which strain E18 makes PRH is unclear. The biosyntheses of other poly (amino acid)s such as ePL and PGA have been catalyzed by membrane bound enzymes [13,14]. Since PRH synthesizing enzymes also might be membrane bound enzymes, it would be difficult to purify the enzymes and to clone these genes. Therefore, it is difficult to improve PRH production by genetic engineering. In addition, the culture condition of strain E18 for PRH

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doi:10.1016/j.bej.2008.07.007

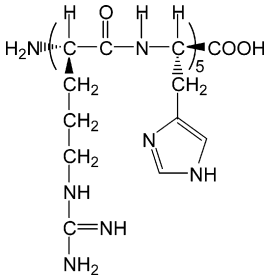

Fig. 1. Structure of poly(arginyl-histidine) (PRH).

production has not been reported yet. In this study, we investigated the culture conditions for PRH production, and collected new information about the effect of culture medium and incubation temperature on PRH production by *V. kibiense* E18.

2. Materials and methods

2.1. Microorganism

The ergot fungus *V. kibiense* strain E18 was kindly provided by Dr. Masanobu Nishikawa, Research Institute for Biological Sciences, Okayama (RIBS). Strain E18 was grown on a potato dextrose agar (PDA) plate at 28 °C, and then maintained at 4 °C for stock culture.

2.2. Medium and culture conditions

For PRH production, *V. kibiense* strain E18 was cultivated in a synthetic glycerol (1Gly1N) liquid medium, that contained glycerol instead of galactose, rather than in a synthetic galactose medium [8], because strain E18 grows faster with glycerol than with galactose. The 1Gly1N medium was composed of 1 L of each of 10 g of glycerol, 0.66 g of ammonium sulfate, 0.88 g of potassium dihydrogen phosphate, 0.25 g of magnesium sulfate heptahydrate, 0.1 g of yeast extract, and 1 ml of Kirk's mineral solution [15]. Strain E18 from the stock culture plate was inoculated and cultivated in 30 ml of 1Gly1N medium in a 100 ml of baffled flask as a preculture. Then the preculture was transferred into a fresh 120 ml of 2Gly2N liquid medium in a 500 ml of Sakaguchi flask as a PRH production culture. The 2Gly2N medium contained carbon and nitrogen source at twice the concentration of the 1Gly1N medium. Strain E18 was cultivated under various conditions, i.e. media composition, initial pH, shaking speed, temperature, and cultivation days.

2.3. Analytical methods

The PRH and ePL secreted into the culture were purified by solid phase extraction (SPE) using a Sep-Pak CM cartridge (cation-exchange type, Waters, Milford, MA). The culture broth was filtered through a micropore membrane to remove cells. The pH of the filtrate was adjusted to 7.0 with NaOH. An aliquot of the sample was then applied to the Sep-Pak CM cartridge equilibrated with MilliQ. The cartridge was washed with 0.2 M acetic acid, PRH, and ePL were eluted with 0.1 M HCl.

An aliquot of 10 μl of the sample was subjected to HPLC (La Chrom Elite, Hitachi, Tokyo, Japan) equipped with a column of μ-Bondasphere C18 (5 μm, 300 Å, 150 mm × 3.9 mm, Waters). The column temperature was maintained at 40 °C. The mobile phases were (A) 0.1% TFA and (B) 0.1% TFA/95% acetonitrile. The gradient elution was performed with a flow rate 0.5 ml/min as follows: 0–10% B for 10 min; 20% B for the next 20 min; 100% B for the next 5 min; and 0% B for the next 15 min. The peptides, PRH and ePL, were monitored by measuring absorbance at 200 and 220 nm.

The concentrations of sugar and organic acids were measured by an HPLC equipped with a column of Shim-pack SPR-H (250 mm × 7.8 mm, Shimadzu, Kyoto, Japan) and with two detectors, an RI detector for sugar as a carbon source and a UV (210 nm) detector for organic acids as metabolic intermediates. The column temperature was maintained at 40 °C. The mobile phase was an aqueous solution of perchloric acid (pH 2). The isocratic elution was performed for 20 min with a flow rate 0.6 ml/min.

All data are mean results of the analysis of duplicate samples. PRH and ePL productivities included deviation scores. The average standard deviation for dry cell weight (DCW), residual carbon, and pH were 5% or less, so these numbers were not taken into account.

3. Results and discussion

3.1. Basal culture condition

The basal culture condition was investigated to determine if it could stably produce PRH by strain E18. We investigated the shaking speed at 0, 60, 90, 100, 110, and 120 rpm on the production culture, and found that PRH production was the highest at 90 rpm. The PRH productions were unstable in the cultures with high reciprocal shaking speeds, but became stable at 90 rpm. In addition, filament shape cells were observed in the cultures with shaking speeds higher than 100 rpm, and these culture broths were turbid. The cells were rigid at lower than 90 rpm, and bead shape cells were observed at 60 and 90 rpm. At 0 rpm, that is static culture, the cell was pellet shaped on the flask wall and sheet shaped on the upper surface of the broth. The culture broths were transparent at shaking speeds lower than 90 rpm. It was suggested that the cell became filament shaped because of the shear stress resulting from the high speed shaking. Furthermore, cell growth increased with an increase in shaking speed. Despite the good cell growth, PRH production of the filament shaped cell was lower than the productions of other shapes. The PRH productions of pellet and sheet shaped cells in static culture were higher than that of the bead shaped cell at 60 rpm, but only one half of the PRH production at 90 rpm. The cell growth in static culture was slightly better than that at 60 rpm, but only approximately two-thirds of the cell growth at 90 rpm. Therefore, high and stable PRH production might be directly related to low cell damage and high cell growth.

Strain E18 was cultivated in the culture medium with the initial pH adjusted from 3.0 to 5.0. There was no remarkable effect. Since the 1Gly1N medium showed approximately pH 5.0 when the pH was not adjusted, the cultivations were performed at an initial pH of 5.0.

As suggested above, cell damage and growth are related to PRH production; therefore, the conditions of preculture were also investigated. The preculture was performed under the combination of media (1Gly1N or 2Gly2N), shaking speeds (90 or 120 rpm), and cultivation days (5 or 7 days). Each preculture was inoculated to a fresh 2Gly2N medium, and cultivated for 7 days with reciprocal shaking at 90 rpm. As shown in Table 1, though there was no remarkable effect, the optimal preculture conditions of medium, shaking speed, and cultivation days were 2Gly2N, 90 rpm, and 7 days, respectively. In fact, the combination of these preculture conditions led to the strain E18 that showed the highest PRH production.

Based on these results, the basal conditions for preculture were determined as follows: medium, 2Gly2N (pH 7.0); rotary shaking, 90 rpm; temperature, 28 °C; cultivation days, 7. The basal conditions for PRH production were determined as follows: medium, 2Gly2N (pH 5.0); reciprocal shaking, 90 rpm; temperature, 28 °C; cultivation time, 7 day.