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Cellular biocompatibility of cyanophycin substratum prepared with recombinant *Escherichia coli*

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ABSTRACT

Cyanophycin from recombinant *Escherichia coli* is composed of aspartic acid as a backbone with arginine and lysine as the side chains. Cyanophycin exists in insoluble and soluble forms based on its solubility in aqueous solution. This study aims to assess the physical properties and cellular biocompatibility of cyanophycin prepared with recombinant *E. coli*. The decomposition temperature of cyanophycin was around 230 °C for both forms of cyanophycin, as measured by thermogravimetric analysis. Soluble cyanophycin showed no toxicity to Chinese Hamster Ovary (CHO) cells at a concentration of 5 mg/mL as revealed by the thiazolyl blue tetrazolium bromide method. When the insoluble cyanophycin formed thin films, the films exhibited a structure of stacking lamellae. CHO cells grown on the films had a higher relative cell density, or 107–142% that of those grown on tissue culture polystyrene (TCPS), 48 h after seeding. After the removal of serum-containing medium, the CHO cells maintained cell morphology for up to 72 h in Dulbecco's modified Eagle medium without serum, and the relative cell density was 150–170% that of the cells grown on TCPS 48 h after serum removal, indicating that the cyanophycin substratum could provide sustained cell growth. When RAW 246.7 cells were grown on the films of insoluble cyanophycin for 96 h, nitric oxide concentration released from the macrophages was below 2 mM/mg protein, suggesting that a minimal immune response was elicited. The results showed that cyanophycin might have the potential to serve as a biocompatible, degradable material in biomedical applications, such as tissue engineering and drug delivery.

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1. Introduction

Cyanophycin (multi-L-arginyl-poly-L-apspartic acid) is a non-ribosomal polypeptide that exists as granules in inclusion bodies mostly inside cyanobacteria [1] and serves as an intracellular energy nutrient when the organisms are grown under nitrogen limitations [2–4]. Cyanophycin has a comb-shaped molecular structure. The backbone consists of aspartic acid of which β-carboxylic group is linked to the amine group of arginine [5,6]. The synthesis of cyanophycin is directed by a single enzyme, cyanophycin synthetase [7–9]. Recently, cyanophycin has been produced by recombinant DNA technology in other organisms such as *Escherichia coli* [8,10–12], *Saccharomyces cerevisiae* [13], and *Pichia pastoris* [14]. *Corynebacterium glutamicum*, *Ralstonia*, *Eutropha*, *Pseudomonas putida* [15], and plants [16,17].

Cyanophycin has been shown to be resistant to several commercially available endoproteases and exopeptidases [9]. However, cyanophycin could be degraded into aspartic acid-arginine dipeptides and free arginine extracellularly by cyanophycinase from cyanobacteria. Several previous studies reported that cyanophycinase from other strains also has the capability of digesting cyanophycin extracellularly [18–21].

The cyanophycin in nature contains an equal molar ratio of aspartic acid and arginine, and has a molecular weight distribution from 25 to 100 kDa [22,23]. The recombinant cyanophycin, with a less dispersed distribution of molecular weight from 14 to 45 kd, can incorporate lysine into the side chains during its synthesis [13,24]. According to the solubility at physiological pH (pH 7), cyanophycin can be classified as soluble and insoluble forms. The soluble form from recombinant *E. coli* has a high lysine/arginine molar ratio and a lower molecular weight distribution than the insoluble form [25]. The primary amine of lysine residues can also allow reactions under moderate conditions, such as at room temperature.

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Several previous studies have demonstrated the applications of cyanophycin [26–28]. The dipeptides produced from the partial digestion of cyanophycin could provide some nutritional benefits [21,27]. However, the use of cyanophycin as a biomaterial has received relatively little attention. In this study, we examine the cellular biocompatibility of both soluble and insoluble forms of cyanophycin. Cyanophycin was prepared with the culture of recombinant *E. coli* harboring the gene coding for cyanophycin synthetase. The powder of purified cyanophycin was subjected to thermogravimetric analysis. Cellular cytotoxicity of cyanophycin was examined by the MTT method for the CHO cells grown in the medium containing soluble cyanophycin, and on the films of insoluble cyanophycin, respectively. The release of nitric oxide from RAW 246.7 cells grown on the films of insoluble cyanophycin was used to assess the immune response.

2. Materials and methods

2.1. Materials

Both soluble and insoluble forms of cyanophycin were prepared with the culture of recombinant *E. coli* harboring cyanophycin synthetase gene on pET21b as previously described [25]. Fetal bovine serum, Penicillin–Streptomycin–Amphotericin B antibiotic solution, and Dulbecco's modified Eagle medium (DMEM) were obtained from Thermo Fisher Scientific (Waltham, MA). Multi-well microplates were from Corning (Tewksbury, MA). Other chemicals were purchased from Sigma–Aldrich (St. Louis, MO) and used as received. Water was deionized by a Milli-Q water purification system (Bedford, MA).

2.2. SDS polyacrylamide gel electrophoresis

The purified cyanophycin was mixed with an equal volume of the loading buffer. The mixture was heated in boiling water bath for 5 min, and 20 μL of the mixture was applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.3. Thermogravimetric analysis

Approximately 3–5 mg of cyanophycin powder was placed in an aluminum pan in a thermogravimetric analyzer (PerkinElmer Diamond TG/DTA) operating under 20 mL/min nitrogen flow. After equilibrium at 25 °C for 30 min, the sample was heated to 750 °C at 10 °C/min. The decomposition temperatures were then analyzed.

2.4. Gel permeation chromatography

The molecular weight distribution of soluble cyanophycin was determined by a gel permeation chromatography (GPC) equipped with Ultrahydrogel columns and a refractive index detector (Waters; Milford, MA). An aliquot of 200 μL aqueous solution containing 0.5 mg/mL of purified soluble cyanophycin was injected into the GPC system with pullulan as calibration standards.

2.5. Cell culture

CHO-K1 cells (a Chinese Hamster Ovary cell line, ATCC-CCL-61) and RAW 246.7 cells (a mouse leukaemic monocyte macrophage cell line, ATCC-TIB-71) were maintained at 37 °C, 5% CO_2, and 100% humidity in complete media containing DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% Penicillin–Streptomycin–Amphotericin B antibiotic solution.

2.6. Preparation of insoluble cyanophycin film

Different concentrations of insoluble cyanophycin were dissolved in 0.1 N HCl. An aliquot of 200 μL was then added into each well of 12-well tissue culture plates to obtain four different films, with cyanophycin concentrations of 0.25, 0.5, 1, and 2 mg/cm^2. After the water was evaporated to dryness at room temperature for 24 h, 1 mL of 0.15 M sodium bicarbonate in 50% ethanol was added into each well to neutralize HCl. Then cross-linking was performed in 1 mL of 70% ethanol containing 0.5% glutaraldehyde for 1 h. After the removal of glutaraldehyde solution, 1 mL of 70% ethanol was added for disinfection for 4 h followed by washing with 1.5 mL phosphate buffered saline (PBS) twice to remove the residual ethanol. The film was further equilibrated with DMEM overnight prior to the application of cells.

2.7. Scanning electron microscopy observation

The films of insoluble cyanophycin were peeled off by forceps, immersed in liquid nitrogen, and snapped to smaller pieces. The snapped film was sputtered with platinum, and both the top surface and the cross section were examined under a scanning electron microscope (JSM-6390, JOEL).

2.8. Measurement of cellular viability

Cellular viability was monitored using thiazolyl blue tetrazolium bromide (MTT) [29]. CHO cells were harvested from the T-75 flasks and seeded at a density of 10,000 cells/cm^2. For the cells on the films of insoluble cyanophycin, the medium was replaced with fresh complete medium every 48 h, and was further cultured until the specified time period. For soluble cyanophycin, the medium was replaced with the complete medium containing various concentrations of soluble cyanophycin at 0.001, 0.01, 0.1, 1, and 5 mg/mL 24 h post seeding.

For the measurement of cellular viability, the cells were washed twice with 1 mL phosphate buffered saline (PBS; pH 7.2). An aliquot of 0.5 mL PBS solution containing 0.5 mg of MTT was added to the cultured cells. After 4 h incubation at 37 °C, 1 mL dimethyl sulfoxide was added to solubilize the colored formazan product, and the absorbance was measured at 540 nm by a spectrophotometer (Jasco, Tokyo, Japan).

A correlation of the cell number and the absorbance from MTT assay was established by applying various amounts of cells onto the multi-well plates. A series of two wells containing the same amount of seeding cells were employed in pairs to establish the quantitative correlation. After 24 h culture, one of the wells was subject to MTT assay, and the cells on the other well were trypsinized and counted with a hemocytometer under a microscope.

2.9. Protein determination

The cells were washed twice with PBS, and then incubated with 0.5 mL of lysis buffer (0.2 M Tris–HCl, 1% Triton X-100, 5 mM EDTA, and protease inhibitors, pH 7.8) for 5 min followed by centrifugation at 10,000 × *g*, 4 °C for 10 min to prepare the cell lysate. The protein content of the lysate supernatant was quantitated using the Bradford's method [30].

2.10. NO release measurement

The amounts of NO released from RAW 246.7 cells into medium were measured by 2,3 diaminonaphthalene (DAN) as previously described [31]. Briefly, 200 μL of the culture medium of RAW 246.7 cells was collected and brought to 1 mL with water. The mixture was mixed with 100 μL of 50 μg/mL DAN in 0.62 N HCl. The reaction

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