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Physical and chemical properties, percutaneous absorption-promoting effects of exopolysaccharide produced by *Bacillus atrophaeus* WYZ strain

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ABSTRACT

A high yield of exopolysaccharides bacteria isolated from mangrove system was identified as *Bacillus atrophaeus* by 16S rDNA and named as WYZ strain. An exopolysaccharide (BPS) was obtained from this strain after purification with a yield of about 0.58 g/L. Then some physical and chemical properties of BPS, such as weight average molecular weight (Mw), monosaccharide composition, intrinsic viscosity and water retention capacity were studied. The microstructure (SEM) showed that BPS was porous wound three-dimensional spider web structure. Using BPS as transdermal absorption enhancer, and lidocaine as a test drug, in vitro and local anesthesia in live animals experiments were conducted to explore that the BPS promote lidocaine transdermal absorption and mechanism. In conclusion, the BPS had good water retention capacity and transdermal absorption promoting effect, all of these indicated that BPS has great potential in the field of biomaterials.

1. Introduction

Microbial exopolysaccharide (EPS) was a long-chain, high-molecular-weight polymer secreted by microorganisms into the environment during fermentation. Due to its high safety, low side effects and unique physical and rheological properties, it has been used as emulsifier, gelling agent, thickener, film former and lubricant in petroleum, chemical, food and pharmaceutical industries and many other fields (Freitas, Alves, Carvalheira et al., 2009). In addition, EPS has been shown to have anti-inflammatory, anti-tumor, anti-oxidant and immunomodulatory properties (Liu et al., 2010). Compared with plant-derived polysaccharides, microbial polysaccharides have the advantages of short production cycle, not limited by season, region, pest and disease conditions, strong market competitiveness and wide application. At present, the production of plant and animal origin colloids was not enough to meet the huge market demand (Laws, Gu, & Marshall, 2001).

Studies have shown that different types of bacteria-derived EPS have higher viscosity thickening, gelling, water retention capacity and emulsifying properties than commercial polymers such as guar gum, locust bean gum and gum arabic. For example, xanthan produced by *Xanthomonas campestris* was used as a food thickening and stabilizing agent (Becker, Katzen, Pühler, & Ielpi, 1998). In yogurt production, EPS produced by *Streptococcus thermophilus* could be used as a natural food additive and could enhance the viscosity and texture of the product (Griffin, Morris, & Gasson, 1996). In addition, Prasanna, Bell, and Grandison (2012) found that EPS produced by *Bifidobacterium longum* subsp. *Inantis* CCUG 52486 and *Bifidobacterium infantis* NCIMB 702205 has higher viscosity and emulsifying activity. Han et al. (2015) obtained two EPSs from *B. amyloliquefaciens* LPL061, which had certain thermal stability and had potential applications in the food and medical industry. In recent decades, the discovery of new EPS has become one of the hot topics in microbial research because of its special advantages in biocompatibility and yield (Freitas, Alves, Pais et al., 2009; Raveendran, Poulose, Yoshida, Maekawa, & Kumar, 2013). In addition, due to its good biocompatibility, water retention and film-forming properties, EPS has inherent advantages in medicinal materials such as external use or skin (Moreno, Vargas, Olivares, Rivas, & Guerrero, 1998). At present, quite a lot of drugs were not ideal for transdermal absorption and cannot meet the treatment requirements. Therefore, many researchers were devoted to discovering new and safe transdermal absorption enhancers (Alexander et al., 2012). Up to now, there were not many studies on EPS as topical medical materials and transdermal absorption enhancers. Lidocaine, a commonly used local anesthetic in clinical practice, was not suitable for oral preparations due to its low oral availability and liver first pass effect (Talbi, Brulin, Campo, & Fourniols, 2017). It was soluble in water and has strong and lasting local anesthetic effect and good penetrating ability, it can be injected and transdermal administered, while injection was less safety (Preis et al., 2014). At present, the clinical demand for transdermal formulations of the drug continues were increasing, and correspondingly accelerate the development of transdermal absorption (Xie, Zhang, Xu,

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Sun, & Yang, 2017). Because of the obvious role of local anesthesia, the ability to penetrate the cell, transdermal delivery of a clear effect, test and test convenience of Lidocaine, therefore this study intended to use lidocaine as a transdermal absorption test to study the transdermal absorption enhancement of BPS.

A strain of high yield of exopolysaccharides was screened in this paper, and morphological and molecular of the strain were identified. Exopolysaccharide (BPS) was extracted from this strain, and a series of BPS physicochemical properties, including its weight average molecular weight, monosaccharide composition, intrinsic viscosity, microstructure and water retention capacity were studied. Finally, using BPS as transdermal absorption enhancer, and lidocaine as a test drug, in vitro and local anesthesia in live animals experiments were conducted to explore that the BPS promote lidocaine transdermal absorption and mechanism, and to explore its application in the field of medicine.

2. Materials and methods

2.1. Strains and media

A strain named WYZ with high production of exopolysaccharides isolated from the mangrove system and was fermented in LB medium (peptone 10.0 g/L, yeast 10.0 g/L, glucose 5.0 g/L, pH = 6.5-7.0) and stored at −80 °C.

2.2. Isolation and identification of strains

The routine identification of WYZ strain was performed as described by Sambrook, Fritsch, and Maniatis (1989). The 16S rDNA from the WYZ strain was simultaneously amplified using two pairs of primers (forward primer: 5′-CAAGTCGAGCGGACAGATGGGAGCT-3′; reverse primer: 5′-AGCTCCCATCTGTCCGCTCGACTTG-3′). The above sequence results were aligned using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Multiple alignment was performed using Clustal X1.81 software and phylogenetic trees constructed using MEGA 5.0 (Song, Rong, Zhao, & Chi, 2013).

2.3. Preparation of BPS

The seed culture medium of the WYZ strain was prepared in advance with a 300 ml Erlenmeyer flask containing 100 ml of medium and cultured at 37 °C with shaking at 180 rpm for 10 h. When the $OD_{600\,nm}$ value of the seed liquid reached 0.6–0.8, 75 ml of the seed liquid was transferred to a 2 l fermentor containing 1500 ml of LB medium (pH = 6.5) and fermented at 180 rpm for 40 h at 37 °C. The above fermentation broth was centrifuged at 10,000 × *g* (4 °C, 5 min) and filtered through a microporous filter (0.22 μm). The pH of the filtered fermentation broth was adjusted to about 2.5 with HCl solution (0.1 M) until a large amount of precipitate was produced. After standing at 4 °C for about 6 h, the supernatant was discarded. The lower layer was centrifuged at 8000 × *g* (4 °C, 5 min) and the collected white precipitate was washed three times with distilled water. All the white solids obtained were added to 100 ml of distilled water and adjusted to clear solution (pH 8–9) with 1.0 N NaOH. The solution was lyophilized to give a white, soft solid. The white solid was added to 100 ml of re-steamed ethanol, and centrifuged at 4000 × *g* for 5 min. The bottom precipitate was washed three times with the e-steamed ethanol and then dissolved in 50 ml of distilled water. The solution was adjusted to clear with 1.0N NaOH, dialyzed (Mw: 8000–14,000 Da) for 48 h, and lyophilized to obtain rough polysaccharides. Then fractional purified by DEAE-52 cellulose column and Sephadex G-75 column. In brief, the rough polysaccharide was dissolved in distilled water to a concentration of 10 mg/ml, centrifuged at 5000 × *g* (4 °C, 5 min), and the supernatant was added to a well-balanced DEAE-52 cellulose column. Eluted linearly with 0–1 M NaCl solution and the components were collected by an automatic collector. The content of polysaccharide was determined by phenol-sulfuric acid method. The main components were dialyzed against distilled water for 48 h and then lyophilized. Then further purified by Sephadex G-75 column (16 × 500 mm), eluted with 0.1 M NaCl, collected by automatic collector (Chen et al., 2011), and then the content of polysaccharide was determined by phenol-sulfuric acid method. The collected fraction was dialyzed (Mw: 8000–14,000 Da) against distilled water for 48 h and lyophilized to obtain pure polysaccharide BPS.

2.4. Physical and chemical properties determination

2.4.1. BPS molecular weight, monosaccharide composition

The determination of the weight average molecular weight (Mw), the number average molecular weight (Mn), and the polydispersity index (Mw/Mn) of BPS was performed by gel permeation chromatography (GPC) reported by Prasanna et al. (2012). Analysis of the monosaccharide composition was performed according to the method of Xu, Shen, Ding, Gao, and Li (2011). Briefly, the purified BPS was hydrolyzed with 12 M H_2SO_4 and stirred at 100 °C for 2.5 h. The hydrolyzate was filtered and centrifuged at 4000 × *g* for 10 min. The pH was adjusted to 6.0 with $BaCO_3$. The supernatant was diluted 20-fold and detected on a Dionex system using a high pH anion-exchange chromatography column with pulse current (HPAEC-PAD). Eluted with a mixture of water and 0.2 M NaOH in a volume ratio of 91:9 at a flow rate of 1.0 ml/min. Compared with glucose, galactose, rhamnose, mannose, fucose, xylose, arabinose, galacturonic acid and glucuronic acid (Sigma, ≧99%).

2.4.2. Determination of intrinsic viscosity

BPS, guar gum, xanthan gum and hyaluronic acid (HA) (Sigma) were dissolved in ultrapure water (0.01–0.1%, w/v). The intrinsic viscosity (η) of the four samples was measured using an Ubbelohde viscometer (25 °C) as described by Prasanna et al. (2012). t and t_0 are the average outflow time of the sample solution and water respectively (repeated operation three times). "C" is the concentration of each sample solution. Based on the above data, introduce the "t" and "t_0" values to the Huggins equation and the Kraemer equation:

$$\eta_{sp}/C = [\eta] + k_H [\eta]^2 C \text{ (Huggins equation)}$$

$$(\ln(\eta_{rel}))/C = [\eta] + k_k [\eta]^2 C \text{ (Kraemer equation)}$$

where [η]: intrinsic viscosity; n_{sp} : specific viscosity, n_{rel}: relative viscosity, "k_H" and "k_k" are the Huggins and Kraemer coefficients. With n_{sp}/c and $\ln(n_{rel})/c$ plotted, the intrinsic viscosity [η] can be calculated from the intercept by extrapolating two linear functions to c = 0.

2.4.3. Scanning electron microscopy analysis

BPS was dissolved in distilled water to a final concentration of 10 mg/ml. Lyophilized the samples, then the thin layer samples were observed by scanning electron microscopy (JSM-840, JEOL, Japan) (Prasanna et al., 2012).

2.5. Water retention capacity of BPS

2.5.1. Liquid absorption rate

The method reported by Govin et al. (2016) was used to determine liquid absorption rate. Weigh two copy of 100 mg dry BPS, were filled in two 250 mesh nylon bags made of "tea bags". One bag was immersed in a beaker containing a large volume of deionized water and the other bag into a beaker containing a large volume of saline (0.9% NaCl) for 2 h at 32 °C. After reaching the aspiration balance, lift the "tea bags", suspend it to allow the unabsorbed water to naturally filter out, and weigh the hydrated tea bag after removing the last drop of water with absorbent paper (METTLER TOLEDO EL204 Electronic Analysis Balance). Hyaluronic acid was set for the positive control group. The liquid absorption rate of BPS was calculated according to the following