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Characterization of a microbial polysaccharide-based bioflocculant and its anti-inflammatory and pro-coagulant activity

Chunying Zhong[a,b,1], Gang Cao[a,1], Kuan Rong[a], Zhengwu Xia[a], Ting Peng[a], Honggao Chen[a], Jiangang Zhou[a,*]

[a] School of Environmental Engineering, Wuhan Textile University, Wuhan 430073, China
[b] Hubei Key Laboratory of Purification and Application of Plant Anticancer Active Ingredients, Chemistry and Biology Science College, Hubei University of Education, Wuhan 430205, China

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ABSTRACT

We describe a novel bioflocculant, MBF-15, which is an exopolysaccharide extracted from the alkaliphilic bacterium *Paenibacillus jamilae*. The biophysical characteristics of MBF-15 were determined using Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy. MBF-15 was also evaluated for its biocompatibility by examining its inflammatory, coagulant, and hemostatic properties in vitro and in vivo. Pretreatment of peripheral blood mononuclear cells with MBF-15 inhibited lipopolysaccharide-stimulated expression of inducible nitric oxide synthase, production of nitric oxide, and secretion of pro-inflammatory cytokines, including tumor necrosis factor-α and interleukin-6. In addition, MBF-15 increased both mRNA and protein levels of the anti-inflammatory cytokines transforming growth factor-β and IL-10. The hemocompatibility of MBF-15 was investigated by measuring the hemolysis ratio and clotting times. MBF-15 had high pro-thrombogenic activity but was not hemolytic. In a rat model, MBF-15 showed superior hemostatic properties compared with chitosan. Thus, MBF-15 offers a promising combination of anti-inflammatory and pro-coagulant properties that may be useful for hemostasis in a variety of clinical settings.

1. Introduction

Absorbable hemostatic agents composed of natural and synthetic polymers have become an indispensable part of the surgical armamentarium to manage intraoperative bleeding. The demand for natural polymers, such as collagen, gelatin, chitosan, and starch, for the treatment of both acute and chronic wounds is rising due to their biocompatibility and biodegradability [1]. A variety of formulations of polymers of animal or plant origin have been developed, including hydrogels, membranes, foams, hydrocolloids, and powders [2]. However, no microbial hemocompatible pro-coagulants of commercial value have yet been described.

Microbial flocculants are metabolites of microorganisms and are composed of polysaccharides, proteins, glycoproteins, and proteoglycans [3]. They are a versatile class of novel biomacromolecules with many potential applications in industry, including the precipitation of pharmaceutical proteins, absorption of heavy ions, dehydration of sludge, purification of drinking water, and control of harmful algal blooms [4]. This versatility stems from their high flocculation performance, lack of toxicity, and ecofriendly biodegradation properties [5].

Microbial biopolymers have become of interest as anti-bacterial, anti-viral, and anti-algal agents and as inducers of microbial aggregation and biofilm formation [6]. Microbial flocculants are also used in the food and pharmaceutical industries as viscosifying, emulsifying, and stabilizing agents [7]. Ghosh et al. demonstrated that bioflocculants are nontoxic in Swiss albino mice and have many medical applications [8]. However, there have been no investigations to date of the interactions between bioflocculants and blood, and an evaluation of their cytotoxicity is necessary before they can progress to clinical development. In contrast, the anti-inflammatory activities of exopolysaccharides have been studied extensively. Paynich found that an exopolysaccharide from *Bacillus subtilis* induced anti-inflammatory M2 macrophages, inhibited T cell activation, and suppressed T cell-dependent immune responses [10]. These results suggest that caution should be exercised when testing novel polysaccharides in clinical trials, with particular attention given to their biocompatibility.

* Corresponding author.
E-mail address: wallice24@hotmail.com (J. Zhou).
[1] These authors contributed equally to this work.

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The objective of the present study was to characterize a bioflocculant derived from *Paenibacillus jamilae*, termed MBF-15, using Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy, and analysis of chemical composition. We also examined the pro- and anti-inflammatory effects, hemocompatibility, and hemostatic properties of MBF-15 using in vitro and in vivo assays.

2. Materials and methods

2.1. Isolation and identification of bioflocculant-producing microorganisms

Bioflocculant-producing bacterial strains were isolated from ramie biodegumming wastewater (chemical oxygen demand [Cr] 21,820 ± 400 mg/L, suspended solids 530 ± 140 mg/L, lignose 250 ± 45 mg/L) obtained from the Research Center for Clean Production of Textile Printing & Dyeing in Wuhan, China. Each isolated bacterium was cultured in fermentation medium (10 mL) containing 2.0% w/w sucrose, 0.5% $(NH_4)_2SO_4$, 0.5% K_2HPO_4, 0.5% yeast extract, and 0.1% NaCl (pH 8.0), on a rotary shaker (150 rpm) at 35 °C for 3 days. These procedures were described previously [7].

A kaolin suspension (5 g/L) was used to evaluate the flocculating performance of a train of culture broths. The RK-15 strain had the highest flocculating rate and was selected for further investigation. PCR amplification of the RK-15 16S rDNA gene and purification of the PCR products were conducted by Takara Biotechnology Co., Ltd. [11].

2.2. Bioflocculant production and flocculating activity tests

Aliquots of 3 mL of the activated cultures of RK-15, adjusted to a cell density of 1.8×10^8 colony-forming units per mL, were inoculated into 300 mL of flocculation medium composed of sucrose 25 g/L, $(NH_4)_2SO_4$ 5 g/L, K_2HPO_4 5 g/L, and NaCl 1 g/L. This medium was used to study the kinetics of bioflocculant production at various temperatures (20, 28, and 37 °C) and pH (6, 7, 8, and 9). Bioflocculant preparation was also analyzed using various carbon sources, including fructose, glucose, lactose, sucrose, maltose, and melibiose (2%), and nitrogen sources (beef extract, peptone, urea, KNO_3, NH_4SO_4, and NH_4Cl). After fermentation, the cell-free supernatant was centrifuged at 12,000 × *g* for 10 min at 5 °C. Bioflocculant in the supernatant was purified as previously described [12], and the flocculating activity was determined using a standard jar test [12].

2.3. Physical and chemical analysis of the purified bioflocculant

FTIR analysis was performed using a Thermo-FTIR spectrophotometer (Nicolet 6700, USA) in the spectral range of 4000–400 cm^{-1}. Total carbohydrate was quantified by the phenol-sulfuric acid method with glucose as a standard [13]. The neutral sugar, uronic acid, and amino sugar content of the bioflocculant was measured after hydrolysis with HCl using the anthrone reaction, carbazole-sulfuric method, and Elson–Morgan method, respectively [14]. Total protein was quantified by the Bradford method, with bovine serum albumin as the standard [15]. The monosaccharide composition was analyzed after hydrolysis with 3 M HCl at 100 °C for 24 h by high-performance liquid chromatography (HPLC) with a Brava C18-BDS (25 cm × 4.6 mm) column, using an eluant of 10 mM KH_2PO_4 (pH 3.0) and methanol (5%, v/v) at a flow rate of 0.5 mL/min. The average molecular weight of the bioflocculant was also determined by HPLC. The elemental composition and functional groups were analyzed using X-ray photoelectron spectroscopy (XPS; Thermo ESCALAB 250Xi, USA). A broad scan was carried out for primary elemental component analysis, and a high-resolution scan was then performed to determine the atomic components in a particular spectrum. The C 1 signal peak (284.6 eV) was selected as an internal reference to calibrate the position of other peaks. All XPS spectra were analyzed with Spectral Data Processor software.

2.4. Isolation of PBMCs

PBMCs were isolated from citrated whole mouse blood by Ficoll density gradient centrifugation as described by Andersen [16]. Briefly, acid citrate dextrose-treated blood was obtained from healthy mice, carefully overlaid on Ficoll-Paque, and centrifuged at 600 × *g* for 30 min at 20 °C. After removing the upper layer, the interphase PBMC layer (lymphocytes, monocytes, and thrombocytes) was removed. The cells were re-suspended in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10 mM glutamine. The protocol to obtain mouse blood was approved by the ethics committee of Wuhan Textile University (Permission number: WHFZDX 2017-007). All animals were handled according to the Chinese National Institutes of Health Guide lines for the Care and Use of Laboratory Animals.

2.5. Effects of MBF-15 on the inflammatory response of PBMCs

PBMCs were cultured in 96-well plates at 1×10^5 cells/well and treated with various concentrations of bioflocculant for 24 h. Cell viability was then determined using the CCK-8 assay (KeyGEN, Nanjing, China) according to the manufacturer's protocol.

To determine the effects of MBF-15 on cytokine release, PBMCs were incubated in the presence of 10 or 100 μg/mL bioflocculant for 1 h, and then stimulated with 1 μg/mL of LPS (*Escherichia coli* 0111:B4) for 24 h. Release of the inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-10, and transforming growth factor-β (TGF-β) into cell supernatants was quantified using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols. mRNA levels of inflammatory factors were measured by reverse-transcription quantitative PCR (RT-qPCR). Total RNA was prepared with an RNeasy kit (Qiagen) according to the manufacturer's instructions, and cDNA was synthesized using a RevertAid™ first strand synthesis kit (Fermentas, Burlington, Ontario, Canada). Samples of cDNA were amplified with gene-specific primers using mouse 18S RNA as a reference. The forward and reverse primers were as follows: TNF-α, 5′-GAAAGCATGATCCGCGACGT-3′ and 5′-CGAAGTTCAGT AGACA GAAG-3′; IL-6, 5′-AGTTGCCTTCTTGGGACTGA-3′, and 5′-CCACGA TTTCC CAGAGAACA-3′; TGF-β, 5′-AGACATTCGGGAAG CAGTGC-3′, 5′-AAAGACAGCCACTCAGGCGT-3′; IL-10, 5′-ACTGCTATGCT GCCTGCTCT-3′, and 5′-TTCACCTGGCTGAAGGCAGT-3′; and 18S RNA, 5′-TCAA CTTCGATGGTAGTCGCCGT-3′, and 5′-TCCTTGGATGTGGTAGCCGTTCT-3′. qPCR reactions were performed under the following conditions: 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s.

2.6. Western blot analysis

Total protein samples from PBMCs were extracted using standard protocols [17]. Equal quantities of protein samples (40 μg) were resolved by 10% SDS-PAGE and then electrotransferred to a PVDF membrane (Millipore, USA). The membrane was incubated with 5% non-fat milk for 1 h to block nonspecific binding and then incubated with monoclonal rabbit antibody to mouse iNOS (1:1000, Boster Biological Engineering Co, Wuhan, China) or a monoclonal antibody to mouse β-actin (1:1000, Abcam, Cambridge, UK) as previously described [18]. The membrane was washed and incubated with a horseradish peroxidase-conjugated secondary antibody (1:3000, Abcam, Cambridge, UK) at room temperature for