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Characterization of biocompatible fungi-derived polymers that induce IL-8 production

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Abstract

Sixteen strains of fungi from 15 different genera were isolated from natural habitats in Thailand to study their biopolymers. Polymer production, chemical composition, and physical and biological properties related to their potential as wound dressing materials were investigated. All polymers were shown to be composed of polysaccharides except those produced from *Fusarium coccophilum* BCC2415 which was a protein–polysaccharide complex. Molecular weights of the polymers ranged from 2.8×10^3–1.2×10^7 Da. Apart from the polymers from *Fusarium coccophilum* BCC2415 and *Cordyceps dipterigena* BCC2073 that were only water soluble, those from other fungi were soluble in both water and DMSO. Different biological assays including cytotoxicity tests were conducted to investigate their biocompatibility. To evaluate the potential of these polymers as wound dressing material, the level of interleukin (IL)-8 produced by normal human dermal fibroblasts (NHF) cells exposed to the polymers were determined. Our results indicated that polymers produced by *Akanthomyces pistillariiformis* BCC2694, *Cordyceps dipterigena* BCC2073, *Paecilomyces tenuipes* BCC2656, and *Phytocordyceps* sp. BCC2744 were biocompatible and inducers of high levels of IL-8.

Keywords: Fungi; Biopolymers; Biocompatible; Interleukin-8

1. Introduction

In the last decade interest in polymers produced by microorganisms has significantly increased. Many microorganisms synthesize exopolysaccharides (EPS), which either remain attached to the cell surface or are found in the extracellular medium in the form of amorphous slime (Sutherland, 1998). EPS in their natural environment are thought to play a role in the protection of the microbial cell against desiccation, phagocytosis and phage attack, antibiotics or toxic compounds, predation by protozoans, osmotic stress, as well as in cellular recognition (De Vuyst and Degeest, 1999). EPS occur widely among prokaryotic species, mostly microalgae but are less common among yeasts and fungi (Sutherland, 1990). Some of those isolated from fungi do possess interesting physical and pharmacological properties such as anti-tumor activity which are currently in clinical use (Franz, 1989), and hypoglycemic activity (Kiho, Hui, Yamane, & Ukai 1993). The EPS from fungi are also used as dietary supplements for enhancing stamina and as a remedy for blood circulatory problems (Bae, Sinha, Park, Song, & Yun 2000). In addition, microbial EPS are employed in wide range of industries depending on their different properties (Selbmann, Onofri, Fenice, Federici, & Petruccioli 2002; Sutherland, 1998).

At present, over 11,000 strains of fungi from diverse natural habitats of Thailand have been isolated, identified and deposited at the National Center for Genetic Engineering and Biotechnology (BIOTEC) Culture Collection (BCC). From our preliminary experiments, we have identified a group of fungi especially entomopathogenic fungi that can secrete exocellular substances resulting in increased viscosity of media. The objective of this study

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is to chemically and biologically characterize exocellular biopolymers produced from 15 genera of fungi isolated from different locations and evaluate their potential as wound dressing materials. In this report, we identified a selected group of fungi producing biopolymers that are both biocompatible as well as promoting the production of IL-8, a cytokine responsible for enhancing the wound healing process.

2. Materials and methods

2.1. Microorganisms and culture condition

Sixteen strains of fungi from 15 different genera were selected as a representative from a group of fungi that were fast growing and produced the exocellular substances resulting in increased viscosity of media. Fourteen strains belong to a group of entomopathogenic fungi, except *Cookenia tricholoma* BCC2468 and *Zygosporim masonii* BCC7543 which are a basidomycete fungus and a seed fungus, respectively. They were initially grown on potato dextrose agar (PDA) at 25 °C for 7 days. The agar block (1 cm^3) containing mycelia was cut into small pieces and then transferred to 25 ml potato dextrose broth (PDB) in a 250 ml Erlenmyer flask and incubated at 25 °C on a rotary shaker at 150 rpm for 7 days. Five percent (v/v) of the seed culture was transferred into the 50 ml PDB in a 250 ml Erlenmyer flask and incubated at 25 °C on a rotary shaker at 150 rpm for 21 days.

2.2. Isolation and purification

Mycelia were separated from culture broth by centrifugation at 10,000 × *g* for 20 min. The supernatant was filtered through a membrane filter (Whatman No. 1). Subsequently, culture filtrate was mixed with four volumes of 95% ethanol, stirred vigorously, and stored at −20 °C for at least 12 h. The precipitated polymers were recovered by centrifugation at 10,000 × *g* for 20 min and the supernatant was discarded. The polymers were then lyophilized and kept at −20 °C until further use.

During purification step, the polymer was re-dissolved in distilled water and any insoluble materials were discarded by centrifugation at 10,000 × *g* for 20 min. The supernatant was dialyzed (molecular weight cut off 2,000 Da, Spectrum Laboratories, Inc., USA) against 4 l of distilled water for 24 h and lyophilized until completely dry.

2.3. Analytical methods

2.3.1. Estimation of mycelial growth and polymer production

To determine the rate of mycelial growth and levels of polymer production, representative samples were collected every 3 days. The cells and polymers were separated and purified as described above. The mycelial dry weight was measured after repeated washing of the mycelial pellets with distilled water and dried at 80 °C until achieving a constant weight. The polymer dry weight was measured after lyophilization.

2.3.2. Colorimetric assays

Carbohydrate, uronic acid and amino sugar contents were estimated by the phenol–sulfuric acid, the carbazole–sulfate and the Morgan–Elson techniques using glucose, glucuronic acid and glucosamine as standards, respectively (Chaplin, 1986). The total protein was identified by the Folin–Lowry method using bovine serum albumin as a standard (Plummer, 1978).

2.3.3. Molecular weight determination

Average molecular weight of the polymers was determined with a gel permeation chromatography (GPC). The analysis was carried out using a PL-GPC 110 system (Polymer Lab, UK) equipped with a refractive index (RI) detector and an Ultrahydrogel linear column (300 × 7.8 mm ID, Waters, USA). The universal calibration log (M_p) versus V_R, where M_p is the peak molecular weight, was obtained by using pullulan standards with molecular weights ranging from 5,900 to 788,000. The injection volume was 20 μl and the flow rate of the mobile phase (0.1 M NaOH) was 0.6 ml/min.

2.3.4. Solubility

One milligram of the polymer was tested for its solubility in 1 ml of the following solvents: distilled water, dimethylsulfoxide (DMSO), acetronitrile, ethanol and hexane. Each polymer mixture was vigorously stirred and allowed to stand for 24 h before determining the solubility levels.

2.3.5. Biological activity and IL-8 production determination

2.3.5.1. Assay for anti-viral activity and cytotoxic effects. Antiviral activity was evaluated against herpes simplex virus type 1 (HSV-1, strain HF ATCC VR-260) employing a modified plaque reduction assay (Abou-Karam and Shier, 1990) and the colorimetric method described by Skehan et al. (1990). Briefly, HSV-1 (30 plaque forming units/60 μl of Eagle's minimum essential medium (MEM) supplemented with heat inactivated 10% fetal bovine serum) was mixed with various concentrations of polymers in 10 μl of 10% DMSO. Subsequently, the mixtures were added to 96-well microtiter plates containing 1×10^5 cells/ml (130 μl/well) Vero cells (ATCC CCL-81) and incubated at 37 C for 72 h in a humidified incubator with 5% CO_2. Cells were then fixed with 10% TCA, stained with 0.057% (w/v) sulforhodamine B and the absorbance was measured at 510 nm to determine percent inhibition. Polymers were also tested for cytotoxic effects to Vero cells and anti-cancer cell proliferation effects against BCA cells (human breast cancer cell line), KB cells (human epidermal carcinoma of mouth,

ATCC CCL-17), and small cell lung cancer cells (NCI-H187) using a method described previously (Skehan et al., 1990). Acyclovir was used as a positive control for HSV-1 bioassay with IC_{50} value of 1.5 μg/ml. Ellipticine was used as a positive control for assays of Vero cells, BCA cells, KB cells and NCI-H187 cells and the IC_{50} values were 0.40, 1.46, 1.33 and 0.39 μg/ml, respectively.

2.3.5.2. Assay for anti-fungal activity. Biopolymers were dissolved in appropriate solvents and tested against *Candida albicans* (ATCC 90028) using a modified soluble formazan assay (Scudiero et al., 1988). In brief, 100 μl of 2×10^6 CFU/ml *C. albicans* in RPMI 1640 medium containing 34.53 g/ml 3-(*N*-morpholino) propanesulfonic acid (MOP) was added to each well of 96-well microtiter plates containing 100 μl of polymers in various concentrations. Subsequently, plates were incubated at 37 °C for 4 h and 50 μl of 1 mg/ml 2,3-bis(2-methoxy-4-nitro-5-sulfonylphenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT tetrazolium) and 0.025 mM *N*-methylphenazolium methosulfate (PMS) was added. After 4 h of incubation at 37 °C, the absorbance at 450 nm was determined to indicate activity of living cells. The IC_{50} value of the positive control in our system, Amphotericin B, was 0.01 μg/ml.

2.3.5.3. Determination of IL-8 production. Normal dermal human fibroblasts were seeded in 96-well microtiter plates at a concentration of 3×10^3 cells/100 μl/well in Dubelco's modified Eagle medium (DMEM) supplemented with heat inactivated 10% fetal bovine serum and incubated at 37 °C in a humidified incubator with 5% CO_2. After 48 h, the medium was replaced with fresh medium containing various concentrations of polymers and incubated for an additional 48 h. Media were then collected and the levels of IL-8 were determined using the h-IL-8 ELISA kit (Roche Applied Science, Germany).

3. Results and discussion

3.1. Mycelial and polymer productivity of 16 representative fungi

Phytocordyceps sp. BCC2744 gave the highest mycelial yield (11.9 g/l) after 15 days of cultivation while *Hypocrella tamurai* BCC2350 produced the lowest mycelial dry weight (5.3 g/l) when compared to other fungi (Table 1). Most of the fungi could produce polymers between 0.3–3.1 g/l. However, the highest polymer yield at 15.71 g/l was produced by *Cordyceps nipponica* BCC2092 after cultivation for 18 days, which was approximately 5 fold higher than the polymer produced by the next highest polymer producer, *Hymenostilbe* sp. BCC2146. The final pH after 21 days of cultivation ranged between 4.99–8.22.

3.2. Chemical composition and properties of the polymers from 16 selected fungi

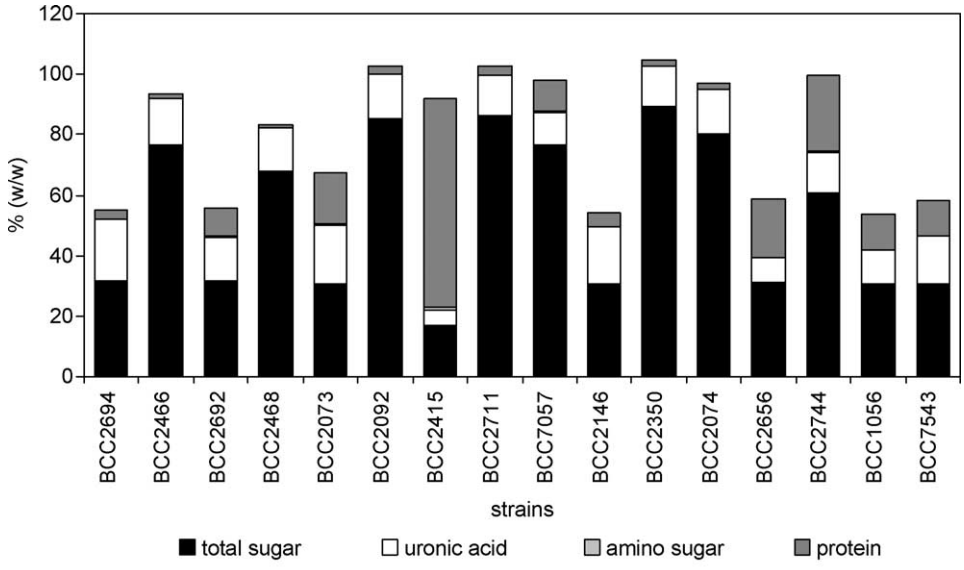
The results indicated that, apart from *Fusarium coccophilum* BCC2415, all studied fungi produced polymers which were mainly composed of sugar and therefore defined as polyssacharides (Fig. 1). In addition, some of these polysaccharides contained an acidic sugar, uronic acid, while amino sugar was not significantly detected (data not shown).

From the solubility test (Table 2), the polymers were insoluble in several solvents which possess less polarity

Table 1
Maximum mycelial and polymer yields of 16 selected fungi

Fungal name and BCC number	Dry cell weight (g/l)[a]	Polymer (g/l)[a]	Final pH
Akanthomyces pistillariiformis BCC2694	10.43±0.32 (12)	2.15±0.05 (3)	6.56±0.11
Aschersonia samoensis BCC2466	5.75±1.09 (18)	2.56±0.03 (6)	5.14±0.13
Beauveria bassiana BCC2692	8.40±0.34 (15)	0.59±0.15 (21)	7.17±0.19
Cookenia tricholoma BCC2468	7.45±0.12 (15)	1.59±0.29 (3)	7.09±0.20
Cordyceps dipterigena BCC2073	9.65±0.12 (12)	2.53±0.52 (6)	6.47±0.06
Cordyceps nipponica BCC2092	10.90±0.81 (21)	15.71±0.51 (18)	6.83±0.05
Fusarium coccophilum BCC2415	9.31±0.01 (15)	2.83±0.46 (9)	7.50±0.16
Gibellula pulchra BCC2711	6.28±0.23 (18)	2.49±0.23 (9)	5.22±0.21
Hirsutella sp. BCC7057	5.57±0.11 (15)	2.78±0.07 (3)	5.12±1.22
Hymenostilbe sp. BCC2146	8.58±0.46 (15)	3.10±0.37 (18)	7.14±0.06
Hypocrella tamurai BCC2350	5.29±0.27 (18)	3.07±0.17 (6)	4.99±0.05
Metarhizium anisopliae var. *majus* BCC2074	7.31±0.67 (15)	2.32±0.67 (6)	7.07±0.01
Paecilomyces tenuipes BCC2656	9.96±0.12 (21)	0.41±0.09 (18)	5.88±0.26
Phytocordyceps sp. BCC2744	11.92±0.15 (15)	2.89±0.06 (3)	5.04±0.64
Torrubiella tenuis BCC1056	7.89±0.52 (9)	0.35±0.02 (18)	7.78±0.09
Zygosporium masonii BCC7543	9.75±0.45 (15)	1.00±0.16 (3)	8.22±0.07

Number in parenthesis indicates number of days in cultivation.
[a] Values are mean±S.D. of three replicates.



BCC2694: *Akanthomyces pistillariiformis*
BCC2466: *Aschersonia samoensis*
BCC2692: *Beauveria bassiana*
BCC2468: *Cookenia tricholoma*
BCC2073: *Cordyceps dipterigena*
BCC2092: *Cordyceps nipponica*
BCC2415: *Fusarium coccophilum*
BCC2711: *Gibellula pulchra*
BCC7057: *Hirsutella* sp.
BCC2146: *Hymenostilbe* sp.
BCC2350: *Hypocrella tamurai*
BCC2074: *Metarhizium anisopliae* var. *majus*
BCC2656: *Paecilomyces tenuipes*
BCC2744: *Phytocordyceps* sp.
BCC1056: *Torrubiella tenuis*
BCC7543: *Zygosporium masonii*

Fig. 1. Composition of the polymers produced from 16 selected fungi.

than DMSO. They were soluble in water and DMSO at different levels, except those from *Cordyceps dipterigena* BCC2073 and *Fusarium coccophilum* BCC2415 which were only soluble in water.

The average molecular weights (M_p) of the polymers determined by GPC were in the range of 2.8×10^3–1.2×10^7 Da. *Fusarium coccophilum* BCC2415 and *Hirsutella* sp. BCC7057 gave the lowest and the highest molecular weight polymers, respectively (Table 2).

When tested for biological activities, the polymer produced from *Fusarium coccophilum* BCC2415 showed anticancer (NCI–H187) and antifungal (*Candida albicans*) activities. Anti-HSV-1 activity was exhibited in polymers produced from *Cookenia tricholoma* BCC2468, *Cordyceps*

Table 2
Molecular weights and solubility of the fungal polymers

Polymers produced from	MW (kDa)	Solubility	
		Water	DMSO
Akanthomyces pistillariiformis BCC2694	8.30	++	++
Aschersonia samoensis BCC2466	114.70	+	++
Beauveria bassiana BCC2692	13.80	++	+
Cookenia tricholoma BCC2468	28.50	+	++
Cordyceps dipterigena BCC2073	20.10	++	−
Cordyceps nipponica BCC2092	208.30	+	++
Fusarium coccophilum BCC2415	2.80	+	−
Gibellula pulchra BCC2711	73.20	+	++
Hirsutella sp. BCC7057	11941.00	+	++
Hymenostilbe sp. BCC2146	161.40	++	++
Hypocrella tamurai BCC2350	99.90	+	++
Metarhizium anisopliae var. *majus* BCC2074	120.40	+	++
Paecilomyces tenuipes BCC2656	4.80	++	+
Phytocordyceps sp. BCC2744	9162.00	+	++
Torrubiella tenuis BCC1056	21.80	++	++
Zygosporium masonii BCC7543	23.20	++	++

++soluble; +partially soluble; −insoluble.

dipterigena BCC2073, *Hirsutella* sp. BCC7057, *Metarhizium anisopliae* var. *majus* BCC2074, *Paecilomyces tenuipes* BCC2656 and *Zygosporium masonii* BCC7543. When normal dermal human fibroblasts (NHF) were exposed to the polymers, IL-8 was produced in culture media at rates ranging from 0.5 to 2.4 ng/ml. The cytotoxicity of these EPS was also studied in two different kinds of mammalian cell lines: Vero and NHF. The polymers produced from *Cookenia tricholoma* BCC2468, *Hirsutella* sp. BCC7057, *Hymenostilbe* sp. BCC2146, *Hypocrella tamurai* BCC2350, *Metarhizium anisopliae* var. *majus* BCC2074 and *Torrubiella tenuis* BCC1056 inhibited growth of NHF, whereas those from *Fusarium coccophilum* BCC2415 were toxic to both Vero and NHF cells (Table 3).

Currently, chitin, a polysaccharide found in arthropod exoskeletons and fungal cell walls, and its derivatives such as chitosan have been widely investigated for applications as alternative biomaterials that accelerate the wound healing process (Mori et al., 1997; Ueno et al., 2001). Chitin and its derivatives have been shown to possess positive effects on the wound healing process by inducing production of different types of cytokines including IL-8, a cytokine that recruits neutrophils to the wounded site as well as stimulates vascularization (Mori et al, 1997; Strieter et al., 1992).

Our results indicated that the 16 fungi produced different levels of exopolysaccharides. From the composition analysis, it was concluded that polymers produced from the studied fungi except *Fusarium coccophilum* BCC2415 are polysaccharides which contain a variety of sugars and protein. The polymer produced by *Fusarium coccophilum* BCC2415 was classified as a polysaccharide-protein complex due to the high protein content (69.22%) detected in the assay. These results corresponded well with previous work which reported that biopolymers from microorganisms can be substituted with pyruvate, acetate, formate, sulfate, phosphate and other groups (Sutherland, 1990). They can also contain a wide variety of sugars and, in some cases, several non-carbohydrate side groups such as fatty acids, proteins, hydrocarbons and other polymers of various sizes, as found in polymers produced from prokaryotic cells (Weiner, 1997).

When tested for cytotoxic effects, 9 fungal strains were shown to be non-toxic to human and primate cells while showing no growth enhancing activities (data not shown). These 9 strains induced IL-8 production at different levels with 6 strains producing IL-8 at rates higher than 0.5 ng/ml, above the level reported from normal human adult dermal fibroblasts but similar to levels produced when cells were treated with platelet-derived growth factor (Liechty, Crombleholme, Cass, Martin, & Adzick 1998). Four of the 9 fungal strains, *Akanthomyces pistillariiformis* BCC2694, *Cordyceps dipterigena* BCC2073, *Paecilomyces tenuipes* BCC2656, and *Phytocordyceps* sp. BCC2744 induced IL-8 production at a level higher than 2.43 ng/ml (a maximum concentration allowed in our test system). It was reported that the blister fluids from burns of human skin produced IL-8 at the level of approximately 10 ng/ml

Table 3
Biological properties of the fungal polymers

Polymers produced from	Biological activity	Cytotoxicity[a]		IL-8 production (ng/ml)
		Vero cell line	NHF	
Akanthomyces pistillariiformis BCC2694	–	−	−	>2.43
Aschersonia samoensis BCC2466	–	−	−	2.30
Beauveria bassiana BCC2692	–	−	−	1.58
Cookenia tricholoma BCC2468	**	−	+	>2.43
Cordyceps dipterigena BCC2073	IC_{50}=47.2 μg/ml against HSV-1	−	−	>2.43
Cordyceps nipponica BCC2092	–	−	−	0.14
Fusarium coccophilum BCC2415	IC_{50}=1 μg/ml against NCI–H187 IC_{50}=7.2 μg/ml against *C. albicans*	+	+	0.61
Gibellula pulchra BCC2711	–	–	−	0.46
Hirsutella sp. BCC7057	**	–	+	>2.43
Hymenostilbe sp. BCC2146	–	–	+	0.18
Hypocrella tamurai BCC2350	–	–	+	2.04
Metarhizium anisopliae var. *majus* BCC2074	**	–	+	>2.43
Paecilomyces tenuipes BCC2656	IC_{50}=34.2 μg/ml against HSV-1	–	−	>2.43
Phytocordyceps sp. BCC2744	–	–	−	>2.43
Torrubiella tenuis BCC1056	–	–	+	0.52
Zygosporium masonii BCC7543	**	–	–	0.13

−nontoxic; **low level of activity against HSV-1.
[a] +toxic/inhibit growth >25% at 50 μg/ml.

(Ono, Gunji, Zhang, Maruyama, & Kaneko, 1995). Therefore, IL-8 induction by these polymers should exhibit beneficial effects to the healing process relevant to actual physiological conditions. Similarly, chitin and its derivatives were shown to induce cytokine production in mammalian cells, but did not stimulate cell proliferation, and this is believed to accelerate wound healing (Mori et al., 1997).

Regarding the level of polymer production, *Akanthomyces pistillariiformis* BCC2694, *Cordyceps dipterigena* BCC2073 and *Phytocordyceps* sp. BCC2744 produced polymers at a similar level, 2.15, 2.53 and 2.89 g/l, respectively, while *Paecilomyces tenuipes* BCC2656 produced polymers at a much lower level (0.41 g/l). However, this level of productivity was obtained from standard culturing conditions using PDB without optimization.

4. Conclusion

We have studied the polymers produced from 16 strains of fungi representing 15 genera and have identified those of three strains which are biocompatible and produce a cytokine that is known to stimulate the wound healing process and have characterized basic chemical and physical properties of these polymers. These three fungal polymers warrant further investigation for future development into a wound dressing materials.

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