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Purification of a fucoidan from kelp polysaccharide and its inhibitory kinetics for tyrosinase

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ABSTRACT

High-speed countercurrent chromatography (HSCCC) was used to separate kelp polysaccharide. HSCCC was performed using an aqueous two-phase solvent system composed of PEG1000–K_2HPO_4–KH_2PO_4–H_2O (0.5:1.25:1.25:7.0, w/w) by eluting a lower aqueous phase at 2.0 mL/min at 600 rpm, yielding two separate fractions, KPS-1 and KPS-2. The KPS-2 fraction was further purified by DEAE-Sepharose fast flow anion-exchange column chromatography to provide 3 fractions, KPS-2-1, KPS-2-2 and KPS-2-3. GPC–HPLC analysis indicated that KPS-2-1 fraction was a purified fucoidan. FT-IR analysis showed that KPS-2-1 was a sulphated polysaccharide. An analysis of enzymatic kinetics showed that the purified fucoidan was a competitive inhibitor of tyrosinase toward L-tyrosine, and the inhibitory constant K_i obtained from double-reciprocal plots was 0.9907 mg/mL.

1. Introduction

Fucoidan is a complex sulphated polysaccharide that is mainly derived from marine brown seaweed. Usually, fucoidan contains large proportions of L-fructose and sulphate (Bilan, Grachev, Shashkov, Nifantiev, & Usov, 2006; Duarte, Cardoso, Noseda, & Cerezo, 2001). Previous studies have shown that fucoidan has a wide variety of bioactivities, such as inflammatory modulation and anti-virus and anti-tumor activities (Alekseyenko et al., 2007; Chandía & Matsuhiro, 2008; Cumashi et al., 2007; Hayashi, Nakano, Hashimoto, Kanekiyo, & Hayashi, 2008; Yang, Aisa, & Ito, 2009). Recently, a fucoidan from algae *Fucus vesiculosus* has been demonstrated to inhibit human immunodeficiency virus (HIV) *in vitro* and shows a synergistic effect with azidothymidine (Chotigeat, Tongsupa, Supamataya, & Phongdara, 2004; Sugawara, Itoh, Kimura, Mori, & Shimada, 1989). Brown seaweed kelp (*Laminaria japonica*) is one of the most important economic seaweeds cultured in China, Japan and Korea, and is widely consumed as a marine vegetable in these countries (Gao, Qin, & Zhang, 2006; Pang, 2007; Suzuki, Furuya, & Takeuchi, 2006). The use of kelp as a drug and an effective component of cosmetics have been well documented in traditional Chinese medicine (Zhang et al., 2007). Kelp is also used as manure, cattle feed, and food for human consumption, as well as a source of phycocolloids, such as agar, alginic acid and carrageenan (Chapman, 1970).

High-speed countercurrent chromatography (HSCCC) has been widely used for the separation and purification of natural products (Yang et al., 2009). HSCCC is a liquid chromatographic technique that operates under gentle conditions and allows non-destructive isolation, even for labile natural compounds. Due to the absence of any solid stationary phase, adsorptive losses are minimized, guaranteeing a 100% sample recovery (Scharnhop & Winterhalter, 2009). To date, the use of HSCCC to isolate products has focused on small organic compounds, and very few efforts have been made to apply this technique to polysaccharides and fucoidans.

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO) (Burton, 1994; Fox, 1991; Robinson & Eskin, 1991; Sanchez-Amat & Solano, 1997; Wong, 1995), is a copper containing mixed-function oxidase widely distributed in microorganisms, animals and plants. This oxidase catalyzes two distinct reactions of melanin synthesis, the hydroxylation of a monophenol and the conversion of an *o*-diphenol to the corresponding *o*-quinone (López-Serrano, Solano, & Sanchez-Amat, 2004; Lontie, 1984; Swan, 1974). The hydroxylation of L-tyrosine, the initial step in melanin synthesis, is of considerable importance since it is also the initial step in catecholamine synthesis. Alterations in melanin synthesis occur in many disease states. Melanoma specific anticarcinogenic activity is also known being linked with tyrosinase activity (Prezioso, Epperly, Wang, & Bloomer, 1992). Melanins are also found in the mammalian eye and brain. Tyrosinase may play a

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role in neuromelanin formation in the human brain and could be central to dopamine neurotoxicity as well as contribute to the neurodegeneration associated with Parkinson's disease (Xu et al., 1997). Tyrosinase is also responsible for browning in plants and considered to be deleterious to the color quality of plant derived foods and beverages. This unfavorable darkening from enzymatic oxidation generally results in a loss of nutritional and economic values and has been of great concern (Friedman, 1996; Jang, Sanada, Ushio, Tanaka, & Ohshima, 2002; Lin, Qian, & Yang, 2010). Similarly, the unfavorable browning caused by tyrosinase on the surface of seafood products has also been of great concern (Kajiwara, Matsui, Akakabe, Murakawa, & Arai, 2006; Kamkaen, Mulsri, & Treesak, 2007; Ogawa, Perdigao, Santiago, & Kozima, 1984). Therefore, the regulation of the tyrosinase activity by its inhibitors has been the hot topic in recent ten years due to its potential applications in medicine, cosmetics and agriculture. To date, many different tyrosinase inhibitors have been suggested as being potential candidates for whitening agents or bio-insecticides (Huang et al., 2009; Kim & Uyama, 2005; Kubo, Kinst-Hori, & Yokokawa, 1994; Nerya et al., 2003). Thus, it is interesting to investigate if kelp fucoidan or polysaccharide can become a potential tyrosinase inhibitor for the above-mentioned use.

In the present study, HSCCC in combination with DEAE-Sepharose fast flow (F.F.) anion-exchange chromatography was used to purify a fucoidan from kelp polysaccharide. GPC–HPLC and FT-IR spectrum analysis was performed to analyze the characterization of the purified fucoidan. The inhibitory kinetics of the fucoidan for tyrosinase toward L-tyrosine was also investigated.

2. Materials and methods

2.1. Reagents and materials

Kelp polysaccharide was prepared according to our previously reported method (Yu & Chao, 2013) and was kept in our laboratory until use. PEG1000, L-tyrosine and the fucoidan standard sample were purchased from Sigma Co. Ltd. All other reagents were of analytical grade.

2.2. Preparation of the HSCCC sample

Kelp polysaccharide was deproteinated by combining TCA with the savage method (Song, Li, & Liu, 2009), and was exhaustively dialysed against water for 48 h. The concentrated dialysate was precipitated with four volumes of absolute ethanol. The precipitate was then washed with absolute ethanol and collected as the refined polysaccharide. The HSCCC sample was prepared by dissolving 1.0 g of the refined polysaccharide into 50 mL of the lower phase of a two-phase solvent system composed of PEG1000–K_2HPO_4–KH_2PO_4–H_2O (0.5:1.25:1.25:7.0, w/w).

2.3. Selection of the solvent system

The first step before performing a separation is to choose a suitable two-phase solvent system by measuring the partition coefficient of the particular target compound. To obtain a suitable solvent system for HSCCC, the partition coefficient K of the sample was detected by TLC. The suitable partition coefficient for HSCCC is approximately $K = 1$, and the sample is soluble in both the lighter and denser phase of the two-phase solvent system (Schwarz, Hillebrand, Habben, Degenhardt, & Winterhalter, 2003). To identify a suitable solvent system, two-phase solvent mixtures composed of PEG1000, K_2HPO_4, KH_2PO_4 and water in different ratios were investigated, and the partition coefficient (K) was determined by TLC. First, 0.5 mg of the refined polysaccharide and PEG1000–K_2HPO_4–KH_2PO_4–H_2O in different ratios were added to a 10 mL test tube with a cap. The sealed tube was shaken vigorously for several minutes to allow complete equilibration. After two clear layers formed, TLC analysis of the polysaccharide components in the two phases was performed. The concentration of polysaccharide components in the two phases was estimated by the color reaction. The partition coefficient for each polysaccharide component in the two phases was calculated.

2.4. HSCCC procedure

HSCCC experiments were performed with a two-phase solvent system composed of PEG1000–K_2HPO_4–KH_2PO_4–H_2O in a ratio of 0.5:1.25:1.25:7.0 (w/w). The upper phase was used as the stationary phase and the lower phase as the mobile phase. The multilayer coiled column was first filled with the upper phase, and the lower phase was pumped into the inlet of the column using a Waters 510 HPLC pump (Millipore Corporation, Milford, MA, United States) at a flow rate of 2.0 mL/min in a head-to-tail elution mode. The apparatus was rotated at 600 rpm. When the front of the mobile phase eluted from the outlet of the column, 1 g of the refined polysaccharide dissolved in 50 mL of the lower phase was injected into the preparative HSCCC system, and the effluent was collected with a BS-100 mode fraction collector (Shanghai Instrument Factory, Shanghai, China). Next, 10 mL of each fraction was subjected to TLC analysis. Fractions comprised of the same polysaccharide component were combined and dialysed with Visking dialysis tubes (molecular weight cut-off of 14,000 Da) to remove the salt and PEG. The resultant polysaccharide component was evaporated under vacuum conditions and freeze-dried.

2.5. Thin-layer chromatography (TLC) analysis of HSCCC fractions

The identity and purity of the obtained HSCCC fractions were determined by TLC. The stationary phase was silica gel 60 F254 from Merck (Darmstadt, Germany), and the mobile phase was *n*-butanol–ethanol–water (5:4:6). After development, the TLC plates were dried and sprayed with an aniline-diphenylamine solution. Blue spots were visible.

2.6. Purification of the KPS-2 fraction

For purification, 50 mg of the KPS-2 fraction was dissolved in 2 mL of distilled water and centrifuged at 10,000 rpm for 10 min. The supernatant was loaded onto a DEAE-Sepharose F.F. anion-exchange column (2.6 cm × 37 cm) pre-equilibrated with distilled water. After the column was equilibrated with the distilled water, a stepwise elution with increasing concentrations of sodium chloride solution (from 0 to 2 M) was carried out at a flow rate of 1.25 mL/min. The eluate was collected using a BS-100 mode fraction collector (Shanghai Instrument Factory, Shanghai, China). The total polysaccharide content in each tube was measured at 490 nm using the phenol–sulphuric acid colourimetric method.

2.7. Homogeneity analysis

Gel-permeation chromatography (GPC) has been shown to be an effective method for the homogeneity determination of the polysaccharide (Lopez-Barajas, Lopez-Tamames, & Buxaderas, 1998). The homogeneity of KPS-2-1, KPS-2-2 and KPS-2-3 was determined by GPC–HPLC. The chromatographic conditions are as follows: Waters HPLC ultrahydrogel linear column (7.8 mm × 300 mm); column temperature, 30 °C; mobile phase, pure water; flow rate, 0.7 mL/min; refractive index detector; detection temperature, 40 °C.