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Mekabu fucoidan: Structural complexity and defensive effects against avian influenza A viruses

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ABSTRACT

Fucoidan from the sporophyll (Mekabu) of brown seaweed *Undaria pinnatifida* (wakame) is interesting due to its various biological activities. Mekabu fucoidan (M_w ~9 kDa) of this study (MF) was previously isolated and characterized by chemical and separation methods including GPC and methylation analysis (Lee, Hayashi, Hashimoto, Nakano, & Hayashi, 2004). It was found that this fucoidan composed of partially sulphated (*DS* ~0.72) fucose and galactose at approximately equal amounts. Methylation analyses revealed complex structure of MF. However, it has been still unclear about the linkages between units and substitution patterns. To solve these structural tasks, spectroscopic methods (FTIR, FT Raman and NMR) were used in the analysis of native MF and its deesterified derivatives. According to obtained results, this polysaccharide was defined as *O*-acetylated sulphated fucogalactan. The defensive effects of MF were evaluated on mice infected with avian influenza A viruses (H5N3 and H7N2 subtypes); its efficacy was determined in reducing viral replication and increasing antibody production. Oral administration of MF resulted in suppressing virus yields. In addition, the production of neutralizing antibodies and mucosal IgA in the animals inoculated with the avian influenza A viruses was significantly increased. These results suggested that MF could be used for the prevention of viral infection.

1. Introduction

Brown alga wakame (*Undaria pinnatifida*) has been traditionally used for preparation of healthy food products in Japan and other countries of East Asia (Taboada, Millán, & Miguez, 2013). This alga is rich of minerals, vitamins, dietary fibres and many other compounds maintaining human health. Partially, its sporophyll (Mekabu) contains polysaccharide fucoidan, which is interesting due to its anticoagulant, immunomodulation, antiviral, antiprotozoal and many other biological activities (Fitton, 2011; Maruyama, Tanaka, Hashimoto, Inoue, & Sasahara, 2007). Fucoidan from *U. pinnatifida* induced apoptosis in various cancer cells via the ROS-mediated mitochondrial pathway (Yang et al., 2013) or through down-regulation of p38, PI3K/Akt and the activation of the ERK1/2 MAPK pathway (Boo et al., 2011). This polysaccharide modulates Th2 responses and thus might be useful for treating allergic inflammation (Maruyama, Tamauchi, Hashimoto, & Nakano, 2005) and mediates tumour destruction through responses of Th1 and NK cells (Maruyama, Tamauchi, Hashimoto, & Nakano, 2003; Maruyama, Tamauchi, Iizuka, & Nakano, 2006). It is less cytotoxic to immune cells than common fucoidan from *Fucus vesiculosis*, and possesses immunomodulating activity to produce cytokines and chemokines from macrophages and splenocytes (Yoo et al., 2007). Mekabu fucoidan showed potential antiviral activities against herpes simplex viruses, human cytomegalovirus and influenza A virus (Hayashi, Lee, Nakano, & Hayashi, 2013; Hemmingson, Falshaw, Furneaux, & Thompson, 2006; Lee et al., 2004). Intake of Mekabu fucoidan was found to increase antibody production in the immunocompromised persons in a randomized, placebo-controlled, double-blind study after influenza vaccination in elderly Japanese men and women (Negishi, Mori, Mori, & Yamori, 2013). This fucoidan also supresses angiogenesis (Liu et al., 2012), demonstrates antioxidant properties (Mak, Hamid, Liu, Lu, & White, 2013) and selectively inhibits several key enzymes including secretory phospholipase A2-IIA, A-kinase and hyaluronidase (Katsube, Yamasaki, Iwamoto, & Oka, 2003; Maruyama, Suzuki, Miyai, &

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Ohtsuki, 2008). Yang et al. (2008) suggested that anticancer activity of Mekabu fucoidan could be significantly enhanced by partial depolymerisation at mild condition. It has been also reported that such low molecular weight fucoidan suppresses inflammation by promoting the inhibition of mitogen-activated protein kinases and oxidative stress (Kim, Yoon, & Lee, 2012). Oligosaccharides obtained by partial enzymatic hydrolysis of Mekabu fucoidan have exerted strong anticoagulant activities by prolongation of activated partial thromboplastin time (APTT) and thrombin time (TT) (Kim et al., 2010).

Influenza epidemics cause numerous deaths and millions of hospitalizations, but the most frightening effects are seen when new strains of the virus emerge, causing worldwide outbreaks of infection (World Health Organization, 2010). Avian influenza is now widespread among poultry and migratory birds in many countries in Southeast Asia and has been detected in Africa and several European countries (World Health Organization, 2013a, 2013b). Defence against influenza infection involves innate and adaptive immune responses. In the previous studies, we have reported that oral administration of the Mekabu fucoidan enhanced innate and adaptive immunities in the host (Hayashi et al., 2013, 2007; Hayashi, Nakano, Hashimoto, Kanekiyo, & Hayashi, 2008). The consumption of the fucoidan has enhanced activity of phagocytic cells, and also improves the function of natural killer (NK) cells (Hayashi et al., 2008). Moreover, adaptive immunity is stimulated as judged from the increase in virus-specific antibody production in mucosa and blood (Hayashi et al., 2013). In addition, sulphated polysaccharides have generally been evaluated as antiviral substances (Ghosh et al., 2009). Therefore, the fucoidan is suggested to be effective in the prevention of influenza virus.

Resent investigations confirmed (Ghosh et al., 2009) that antiviral activities of sulphated polysaccharides depend not only on their chain length and charge density, but also on their specific structural features like branching, sulphation and/or acetylation patterns etc. Unfortunately, the structure of Mekabu fucoidan is still poorly investigated. Fucoidan yield and composition significantly varied with sporophyll maturation (Mak et al., 2013) and cultivation forms (Lee, Lim, Lee, & Park, 2006) of *U. pinnatifida*. In contrast to common fucoidans, this polysaccharide consists mainly of fucose and galactose at various ratios, i.e. it is galactofucan. In addition, two types of acidic groups, i.e. sulphate and acetic acid, were found to be attached to fucose and/or galactose units as fucosyl (galactosyl) esters (Synytsya et al., 2010). The molecular weight and degrees of substitution (sulphatation and *O*-acetylation) are very variable (Hemmingson et al., 2006). Methylation analysis of several preparations confirmed that this polysaccharide has complex structure with various sugar linkages and sulphate substitution patterns (Hemmingson et al., 2006; Lee et al., 2004). However, relationship between the galactan and fucan parts in whole polysaccharide as well as the distribution of sulphate and acetyl groups are still unclear and need more investigation. Spectroscopic methods (FTIR, FT Raman and NMR) have been successfully used in structural analysis of fucoidans (Bilan et al., 2010, 2004, 2002; Pielesz, Biniaś, & Paluch, 2011; Synytsya et al., 2010), and thus these methods are valuable for this purpose.

This work is devoted to structural analysis of the native Mekabu fucoidan and its desulphated and/or deacetylated derivatives by the use of vibration spectroscopy (FT Raman, FTIR) and correlation NMR experiments. This polysaccharide (M_w ~9 kDa) was previously isolated and characterized by chemical and separation methods including GPC and methylation analysis of native and desulphated forms (Lee et al., 2004). However, the used methods were not sufficient to describe its exact structure. The main task of the present work is to determine anomeric configuration and specific substitution of the structural patterns, i.e. fucose and/or galactose units, and confirm glycosidic connections between them. In this work we also investigated the effects of the Mekabu fucoidan on the viral replication and immune responses induced by avian influenza viruses (H5N3 and H7N2 subtypes) in animals.

2. Experimental

2.1. Extraction and purification of fucoidan

Crude fucoidan was isolated from raw material (sporophyll of *U. pinnatifida*) and then purified according to Lee et al. (2004). Previously raw material was homogenized and defatted by ethanol refluxed at 80 °C for 2 h; then polysaccharide was extracted with 0.15 mol l^{-1} HCl. The extract was neutralized, applied to ultrafiltration and then concentrated. The crude polysaccharide was precipitated from concentrated extract by an excess of ethanol, washed and dried. Purification of the fucoidan was made by the subsequent use of DEAE Toyopearl 650 M, Q-Sepharose FF and Sephacryl S300 HR columns. Fractions were collected and checked by the phenol–H_2SO_4 reagent. Most abundant fractions were selected and each case and used for further purification. Finally purified colourless polysaccharide was dialyzed and lyophilized.

2.2. Deacetylation and desulphation of fucoidan

The purified native fucoidan (**MF**) was treated with aqueous ammonia at 37 °C to remove *O*-acetyl groups (Chizhov et al., 1999). Desulphation of **MF** was performed by the solvolytic method (Lee et al., 2004; Nagasawa, Inoue, & Kamata, 1977). Briefly, the sample of fucoidan was passed through Dowex 50 Wx 8 column (H^+ form) with H_2O. After neutralization with pyridine, the solution was lyophilized. The resulting pyridinium salt of fucoidan was dissolved in 10% methanol/DMSO mixture and then incubated at 80 °C for 5 h with continuous stirring. The solution was dialyzed against distilled water and then lyophilized. Obtained deacetylated and desulphated derivatives of **MF** were assigned as **DA-MF** and **DS-MF**, respectively.

2.3. Spectroscopic measurements

FTIR spectra (spectral region 4000–400 cm^{-1}, 64 scans, resolution 2 cm^{-1}) of the fucoidan samples were recorded in the form of KBr tablets on Nicolet 6700 spectrometer (Thermo Scientific, USA) using Omnic 8.0 software. FT Raman spectra (spectral region 4000–100 cm^{-1}, 256 scans, resolution 2 cm^{-1}) of the samples were recorded with Bruker FT Raman (FRA 106/S, Equinox 55/S) spectrometer equipped with Nd:YAG laser (excitation line 1064 nm, laser power 100 mW), quartz beam splitter and Ge detector (cooled with liquid N_2). Obtained spectra were exported to Origin 6.0 (Microcal Origin, USA) software in CSV or TXT format, where they were 10-point filtered and baseline corrected. The second derivative algorithm was used for analysis of overlapped bands. Peak decomposition of FTIR spectra (783–883 cm^{-1}, multiple Voigt functions) was made using peak fitting module of Origin 6.0 (Microcal Origin, USA) software. Areas of the Voigt components were used for calculation of the relationship between specific sulphate esters in fucoidan.

^1H and ^{13}C NMR spectra of native and modified fucoidans were recorded on Bruker Avance 600 and Bruker Avance 500 in D_2O solutions. Working frequencies were 600.1 MHz and 499.8 MHz for ^1H, 150.9 MHz and 125.7 MHz for ^{13}C, respectively. Correlation spectroscopic ^1H, ^1H-PFG-COSY, ^1H-PFG-TOCSY, ^1H-PFG-ROESY, ^1H, ^{13}C-HSQC and ^1H, ^{13}C-HMBC experiments were applied for resolution and assignment of resonance signals.