



EFFECT OF HYDROLYZED FUCOIDAN FROM THE BROWN SEAWEED *Sargassum binderi* SONDER TOWARDS HUMAN BREAST CANCER T47D CELL LINES

Ellya Sinurat^{1*}, Endang Saepudin², and Fildzah Alfita Qosthalani²

¹Research Center for Marine and Fisheries Product Processing and Biotechnology,
Jalan KS Tubun Petamburan VI, Slipi, Central Jakarta 10260, Indonesia

²Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Indonesia

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Abstract

Fucoidan, a sulfated heteropolysaccharide, consists of L-fucose and sulfate ester groups as the main component. Over the past three decades, fucoidan structures and bioactivities have been widely studied. The chemical components (fucose, galactose, small monosaccharides and also the sulfate) and the molecular weights of fucoidans from different brown seaweed species produce different characteristics and structures of fucoidan. The activity of fucoidan against cancer cells has been reported to be affected strongly by their sulfate content and molecular weight. Low-molecular-weight fucoidans tend to have higher solubility and easily penetrate into cancer cells. The objective of this study was to investigate the effect of hydrolyzed of fucoidan on its anti cancer activity against the breast cancer T47D cells. In this study, the fucoidan from the brown seaweed *Sargassum binderi* Sonder was extracted using 0.1 N HCl and was depolymerized by acid hydrolysis at various times and concentrations. Result showed that fucoidan hydrolyzed with 1 M trifluoroacetic acid (TFA) for 1.5 hours reached the maximum depolymerization process and resulted in the decrement of molecular weight from 785.12 kDa to 5.79 kDa as well as sulfate content from 18.63% to 8.69%. The IC_{50} values of fucoidan and low molecular weight fucoidan against the breast cancer T47D cells were 60.03 μ g/mL and 182.34 μ g/ respectively. This result indicated that the sulfate content of fucoidan probably affected its anticancer bioactivities.

Keywords: fucoidan, hydrolysis, brown seaweed, sulfate, anticancer

1. Introduction

Brown seaweed has long been utilized in the industrial field as a source of iodine, minerals, fatty acids, and vitamins (Kim, 2011). Nowadays, brown seaweed has shown a great potential in producing bioactive compounds as new candidates for medicinal uses (Hayes, 2012). Fucoidan is a polysaccharide mostly found in brown seaweed and contains significant amounts of L-fucose with sulfate ester groups. In contrast to other polysaccharides, fucoidan has shown variation in monomer arrangement and sulfate group distribution among different species of brown seaweed. The structural characteristics of fucoidan are likely dependent on the extraction techniques (Bilan et al., 2010; Ponce et al., 2003), seaweed species, harvesting season (Honya, Mori,

Anzai, Araki, & Nisizawa, 1999), geographic location (Rioux, Turgeon, & Beaulieu, 2007), and algal maturity (Zvyagintseva et al., 1999). These factors contribute to the fact that fucoidan structure is complex (Shevchenko et al., 2015). Apart from its complex structures, fucoidan has been found to inhibit the growth of cancer cells (Oliver, Ramon, Andreia, & Tiago, 2017; Vishchuk, Ermakova, & Zvyagintseva, 2013; Usui, Asari, & Mizuno., 1980; Isnansetyo et al., 2017).

The molecular weight and the number of sulfate ester groups, rather than the carbohydrate structure in fucoidan, are the two main parameters in determining the biological activity (Haroun-Bouhedja, Ellouali, Siquin, & Boisson-Vidal, 2000). To increase the anticancer activity, the low molecular weight of fucoidan tends to have a higher solubility that helps

*Corresponding author.
E-mail: ellya_sinurat@yahoo.com

fucoidan to penetrate into the targeted cells. Also, the high amount of negative charge from the sulfate ester groups will presumably strengthen the interaction sulfate in fucoidan with the protein of the cancer cells. Therefore, to study the inhibition ability of fucoidan towards cancer cells, further research in fucoidan depolymerization should be conducted.

Depolymerizing fucoidan from polysaccharides to oligo- and monosaccharides has been done by several techniques, such as by acid treatment (Karlsson & Singh, 1999), enzymatic degradation (Yang et al., 2009) and gamma irradiation (Choi & Kim, 2013). While gamma radiation may be used to depolymerize fucoidan, it requires a sophisticated technology that is too complex for a routine process.

Fucoidan has been widely reported to have an inhibitory activity against cancer cells. The structure and the chemical compositions of fucoidan affect its biological activity. General aspects that influence the fucoidan bioactivity are monosaccharide composition, sulfate content, molecular weight, binding model/ position, and the arrangement of monosaccharide residues. The number of branching sulfate groups also appears to be the key of higher bioactivities fucoidan since it highly affects the negatively charged fucoidan that inhibit the cancer cell proliferation (Oliver, Ramon, Andreia, & Tiago, 2017). In this study, fucoidan depolymerization was carried out by acid hydrolysis method with trifluoroacetic acid (TFA) at various concentrations and incubation times. This study was focused on the effect of hydrolyzed fucoidan towards the inhibition level of human breast cancer T47D cell lines. We used prepurified fucoidan extracted from the same brown seaweeds *Sargassum binderi* Sonder as reference.

2. Material and Methods

2.1. Material

The raw material used in this work was dried brown seaweed with the moisture content of $27.92 \pm 0.35\%$. The brown seaweed (*S. binderi* Sonder) was collected from Lampung, Indonesia. Chemical reagents used were analytical grades and technical grades.

2.2. Extraction, Purification, and Hydrolysis of Fucoidan

About 100 g of cleaned and dried *S. binderi* was ground into small pieces and macerated with 500 mL of a solvent mixture of MeOH – CH₂Cl₂ – H₂O (4:2:1) at room temperature for 3 hours to remove the lipophilic pigments (chlorophyll, fucoxanthin, carotene) and fats. The resulting extract was then treated with 2 L of 0.1

N HCl (1:20) (w/v) at pH 4 and stirred for 6 hours at room temperature. The filtrate was collected and centrifuged at 5,000 rpm for 15 minutes. The filtrate was subsequently neutralized with 0.5 M NaOH, added with 2% CaCl₂ solution and incubated for 3 hours then centrifuged. The filtrate was collected and added to ethanol (1:2) and incubated overnight. The precipitate in the ethanol solution was separated by centrifugation and freeze-dried to obtain crude fucoidan. Purification of fucoidan was conducted using a dialysis membrane (Thermo Scientific Slide-A-Lyzer 3.5 kDa) in a 0.5 M NaCl solvent and continued with aquabidest for 24 hours. About 50 mg of purified fucoidan was hydrolyzed with 5 mL of TFA solution at various concentrations (1, 2, and 3 M) and incubation times (1.0; 1.5; and 2.0 hours) at 121 °C using autoclave. Finally, the fucoidan solution was neutralized with 0.5 M NaOH and then freeze-dried to obtain a hydrolyzed fucoidan (modified method of Sinurat et al., 2015). The hydrolysis of fucoidan was conducted in triplicates.

2.3. Characterization of Purified and Hydrolyzed Fucoidan

2.3.1. Determination of monosaccharide composition

The determination of monosaccharide compositions in hydrolyzed fucoidan was performed using HPLC Shimadzu Prominence-20 instrument with a Hi-Plex H column following Sinurat et al. (2015). The fucoidan solution from hydrolysis treatment was spiked with monosaccharide standards (fucose, galactose, rhamnose, xylose, glucose, and mannose) at varying concentrations (1000, 2000, 3000, 4000, 5000 ppm). About 20 µL of each sample was injected into the column with a flow rate of 0.6 mL/min for 20 minutes using 0.005 M H₂SO₄ as eluent.

2.3.2. FT-IR analysis

The analysis of fucoidan functional group was performed by mixing 2 mg of purified and hydrolyzed fucoidan with 200 mg of KBr (1: 100). The resulting pellet was scanned with FT-IR (Perkin Elmer Spectrum One) for 128 times at 4000-400 cm⁻¹ range.

2.3.3. Determination of sulfate content

The determination of sulfate ester content in fucoidan was performed by BaCl₂ – gelatin method. In 2 mL of 3.5 N HCl, 6 mg of purified and hydrolyzed fucoidan were hydrolyzed for 17-18 hours at 105-110 °C (Dodgson & Price, 1962). Then, 10 µL of hydrolyzed fucoidan was placed into the 96-well plate, added with

190 µL of 3% TCA and 50 µL of 0.5% BaCl₂ – gelatin. The plate was left for 20 minutes until the barium sulfate ready to be scanned. Na₂SO₄ was used as a standard with various concentrations (100 – 500 ppm). Both sample and standard solutions were scanned against blank solution in triplicates with Microplate Reader Spectrophotometer at λ = 360 nm.

2.3.4. Determination of total carbohydrate

The determination of total carbohydrate content was performed by phenol - sulfuric acid method (Dubois Gilles, Hamilton, Rebers, & Smith, 1956). The prepurified and hydrolyzed fucoidan (5 mg) was dissolved in 1 mL of aquadest and then 2.5 mL of concentrated H₂SO₄ was added prior to incubation in an ice tub for 30 minutes. Subsequently, 0.5 ml of 5% phenol was added into the sample solution and was also incubated in the ice tube for 30 minutes. Fucose, mannose, and xylose were used as standards at various concentrations (200, 400, 600, 800, 1000 ppm). Sample, standard, and blank solutions were measured in triplicates at 480 nm and 490 nm with a UV-VIS spectrophotometer.

2.4. Anticancer Activity Assay

2.4.1. Cell line and culture

The breast cancer T47D cells from BBRP2BKP collection was cultured in RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 2% penicillin-streptomycin (Gibco, Grand Island, NY, USA), and 0.5 % fungison (Gibco) in an incubator (ESCO Cell Culture) with a humidified 5 % CO₂ at 37 °C.

2.4.2. Growth inhibition assay

The effect of hydrolyzed and non-hydrolyzed fucoidan on the inhibition of cancer cells was assessed *in vitro* by MTT assay (Isnansetyo et al., 2017). Purified and hydrolyzed fucoidans were respectively dissolved in aquadest, DMSO, and growth medium into different dosages. Three types of control were also prepared, which are cancer cell control, media control, and sample control. Briefly, human breast cancer T47D cells lines were incubated in 96-well plate (1,5 x 10⁴ cells/well) until reaching a confluent phase (12–24 hours). After incubation, 100 µL of serial dosages of purified and hydrolyzed fucoidans were respectively added and incubated for 24 hours in a CO₂ incubator. Cells were then added with MTT reagent and incubated for 4 hours. The reaction was stopped with the addition of 10% SDS and incubated

for 12 hours. The cell growth was observed with microscope inverted and absorbance measured in triplicates with Spectrophotometer Microplate Reader at wavelength 570 nm. The inhibition rate was calculated according to the formula:

$$\text{Inhibition rate (\%)} = \frac{(C-D)-(A-B)}{(C-D)} \times 100 \%$$

where each absorbance stands for: A = fucoidan samples; B = control sample; C = control cells; D = control media. The IC₅₀ value was calculated by probit analysis.

3. Results and Discussion

3.1. Results

3.1.1. Isolation and chemical characteristics of crude and purified fucoidan

The yield of crude fucoidan (CP) obtained from dried brown seaweed *S. binderi* Sonder was 6.64%. After purification with a dialysis membrane (3.5 kDa cut-off), 1.67% of purified fucoidan (PF) was obtained. The total content of carbohydrates and sulfates in both fucoidan species is shown in Table 1. Crude fucoidan (CF) gave a higher yield than the PF, but has a lower percentage of total carbohydrate (47.65%) than PF (82.79%). It indicated that the dialysis process has significantly increased the purity of fucoidan. Hydrolysis of purified fucoidan decreased the sulfate content and also its molecular weight as shown in Table 1.

3.1.2. Fucoidan depolymerization

The oligomer and monomer levels are important parameter to determine the optimum hydrolysis of fucoidan. Lowest oligomer level indicates the most optimum hydrolysis function. The fucoidan that hydrolyzed into monomers, as expected, contained a high level of fucose. Since fucose is the major monomer constituent of fucoidan, its level in fucoidan can be used as indicator of the hydrolysis process level. Figure 1 shows the hydrolysis results, whereas Figure 2 shows the monosaccharide content in fucoidan oligomers after hydrolysis treatment. The optimum hydrolysis was achieved using 1.5 h, 3M TFA and 1.5 h, 1M TFA. We consider using hydrolysis using the lower concentration of TFA, for fucoidan hydrolysis i.e 1M as the best concentration for fucoidan hydrolysis, used for the next study on the basis of economical and reasons.

3.1.3. Chemical characterization of hydrolyzed fucoidan

In hydrolysis treatment, most of the chemical characteristics in fucoidan was affected. In Table 1, it

Table 1. Yield and chemical characteristics of crude and purified fucoidan

Fucoidan	Yield (%)	Total Carbohydrate (%)	Sulfate Content (%)	Mw (kDa)
Crude (CF)	6.64	47.65±1.05	22.99±0.8	1407.67
Prepurified (PF)	1.67	82.79±1.4	18.63±0.7	785.12
Hydrolyzed Fucoidan (1.5 H – 1 M)	42±3.0	55.86±1.2	8.69±0.5	5.79

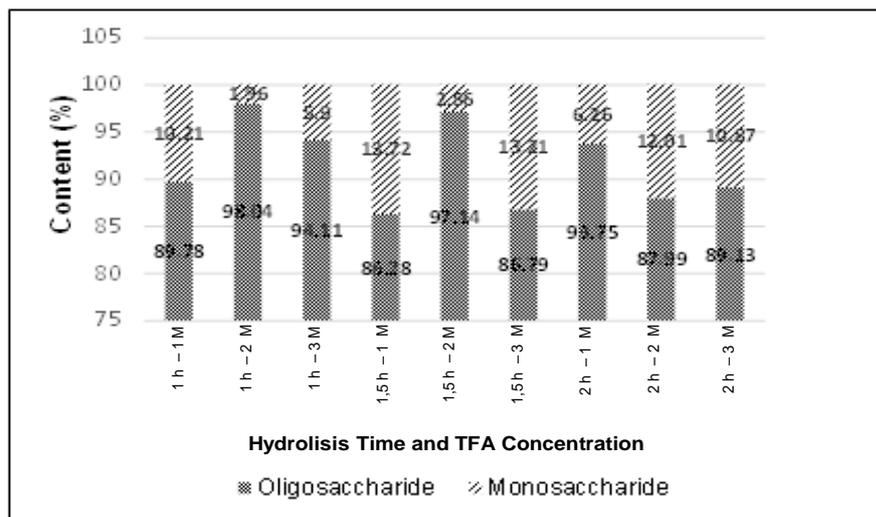


Figure 1. Diagram of fucoidan fragments at various hydrolysis treatment.

is shown that the maximum hydrolysis condition reduced the total carbohydrate and sulfate contents in fucoidan to 55.86% and 8.69%, respectively. The depolymerization process reduced molecular weight of prepurified fucoidan from 785.12 kDa to 5.79 kDa after hydrolysis.

The monosaccharide composition of fucoidan *S. binderi* Sonder determined by HPLC with the RI detector mainly consisted of fucose and mannose. While in the same brown seaweed species (Sinurat et al., 2015), fucoidan from *S. binderi* Sonder has been reportedly composed of fucose, galactose, glucose, mannose, xylose, and glucuronic acid (abundance ratio of 1:2:4:1:1:2) by the GC-MS method. It was achieved after fucoidan was purified in DEAE resin and hydrolysis in 2 M of TFA for 1 hour. Purification and hydrolysis at high temperature cleaved fucoidan and at such condition, some mono and oligosaccharides may undergo decomposition through the so called caramelization.

3.1.4. FT-IR analysis

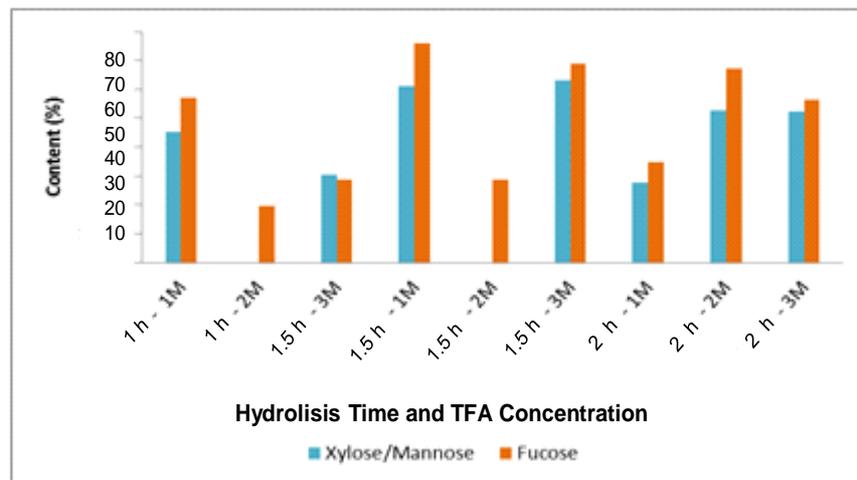
Result FT-IR analysis of fucoidan showed the presence of sulfate ester groups as well as their binding positions. The wavenumber at 1240-1260 cm⁻¹

¹ indicates the presence of symmetric sulfate ester group (O = S = O), while the asymmetric sulfate ester (O-S = O) was detected at 1030 -1060 cm⁻¹. In addition, the ester sulfate binding position can be distinguished to axial (840 cm⁻¹) and equatorial (820 cm⁻¹) positions (Usov & Bilan, 2009).

Based on the FT-IR spectra (Figure 3) and the detail lists on Table 2, the presence of sulfate ester group in crude and prepurified fucoidan appeared in symmetrical, whereas the sulfate ester group in hydrolyzed fucoidan appeared in axial or equatorial. The binding position also showed a different result, where the prepurified fucoidan appeared in equatorial and the hydrolyzed fucoidan in axial. This FT-IR result illustrates that acid hydrolysis treatment might affect the sulfate ester group conformation that potentially influence the bioactivity of fucoidan, i.e, the inhibition against T47D breast cancer cells.

3.1.5. Cytotoxicity of fucoidan against human breast T47D cancer cell lines

The anticancer activity of fucoidan against T47D breast cancer cells was assessed by MTT method and was expressed in IC₅₀ values.



Note: At that condition (1.5 hours incubation, 1 M TFA) the oligomer level was 86.28% containing the fucose level of 7.59%.

Figure 2. Diagram of monosaccharides content at various hydrolysis treatment.

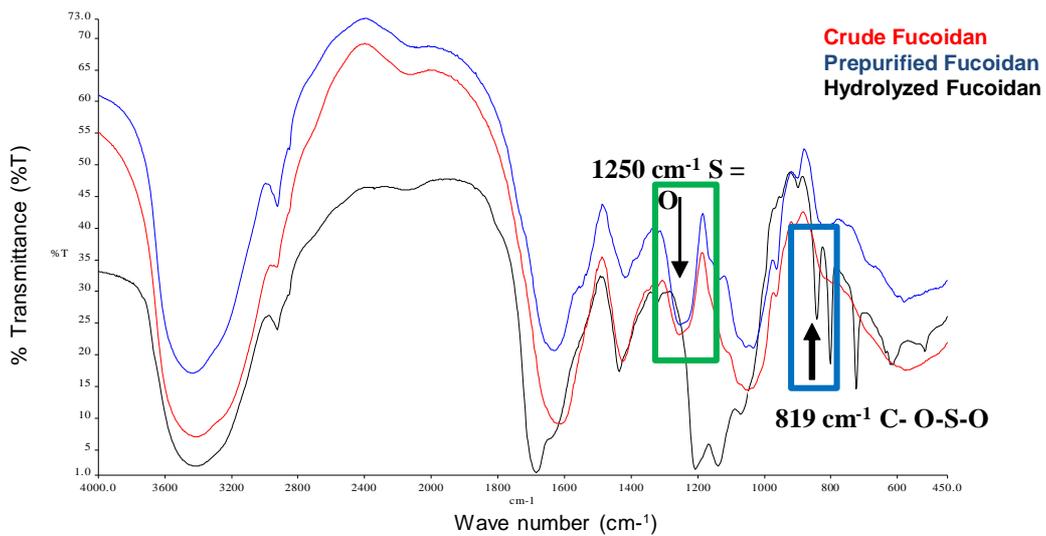


Figure 3. FT-IR spectra of crude fucoidan (red), prepurified fucoidan (blue), and hydrolyzed fucoidan (black).

Table 2. Ester sulfate functional groups of crude, prepurified and hydrolyzed fucoidans

Functional Group	Crude Fucoidan	Prepurified Fucoidan	Hydrolyzed Fucoidan
Sulfate ester symmetric (O=S=O) (cm ⁻¹)	1256.22	1254.22	-
Sulfate ester asymmetric (O-S=O) (cm ⁻¹)	-	-	1071.91
Sulfate ester equatorial (cm ⁻¹)	-	819.13	-
Sulfate ester axial (cm ⁻¹)	-	-	843.12

At a dose of 60.03 µg/mL, prepurified fucoidan inhibited 50 % of the viable T47D cells population. Meanwhile, to reach the same result, hydrolyzed fucoidan needed to triple its concentration as those of the prepurified fucoidan (182.34 µg/mL). This result showed that low molecular weight fucoidan produced by hydrolysis treatment was not as cytotoxic as the prepurified fucoidan. It was assumed that the fucoidan was only partially hydrolyzed. The hydrolysis product lower also has a sulfate content (8.69%) than that of the prepurified fucoidan (18.63%) (Table 1).

Dumitriu (1996) reported that fucoidan with molecular weight ranges of 50 – 100 kDa performed the highest biological activity, while the higher molecular weight (> 850 kDa) fucoidan showed lower activity. Depolymerization play a role in the formation of low molecular weight fucoidan (Chevolot et al., 2001). The high molecular weight fucoidan consists of a long-chain polysaccharide. As a result, it will be more difficult to digest, especially by a low living entity such as cancer cells (Shimizu et al., 2005).

In this study, the inhibition rate of prepurified fucoidan was higher than that of the hydrolyzed fucoidan. It indicates that the molecular weight of fucoidan was not the only factor that affects the cytotoxicity of fucoidan. During depolymerized process by acid hydrolysis, the fucoidan showed a massive reduction in its molecular weight from 785.12 kDa to 5.79 kDa. The sulfate ester group attached to fucoidan might have been also reduced and caused a decreasing concentration of sulfate content. In this case, the hydrolyzed fucoidan contained a lower sulfate content that might cause to a lower inhibition rate to T47D breast cancer cells compared to that of the prepurified fucoidan. Based on this study, the sulfate content has an important role in the fucoidan cytotoxic activity againsts T47D breast cancer cells.

4. Conclusion

The prepurified fucoidan inhibited 50 % of T47D breast cancer cells with lower dosage (60.03 µg/mL) than that of the hydrolyzed fucoidan (182.34 µg/mL). The sulfate content of fucoidan might play a significant role for its bioactivity as anticancer.

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