Solid-Phase Colorimetric Method for the Quantification of Fucoidan

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Solid-Phase Colorimetric Method for the Quantification of Fucoidan

Jung Min Lee · Z.-U. Shin · Gafurjon T. Mavlonov · Ibrokhim Y. Abdurakhmonov · Tae-Hoo Yi

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Abstract We described the simple, selective, and rapid method for determination of fucoidans using methylene blue staining of sulfated polysaccharides, immobilized into filter paper and consequent optic density (at A_{663}nm) measurement of the eluted dye from filter paper. This solid-phase method allows selective determination of 1–20 μg fucoidan in presence of potentially interfering compounds (alginic acid, DNA, salts, proteins, and detergents). Further, we demonstrated the alternative way of using image processing software for fucoidan quantification without extraction of methylene blue dye from stained spots of fucoidan–dye complex.

Keywords Sulfated polysaccharides · Fucoidan · Methylene blue binding · Solid-phase colorimetry · Spot quantitation · Quantity One® soft

Introduction

Fucoidans are group of water-soluble sulfated polysaccharides of sea algae that were discovered almost 100 years ago [1]. Interests to fucoidan, its molecular structure, and biological activity are increasing [2]. Growing research interest underlies necessity of developing of a method for fucoidan quantification with improved selectivity, high sensitivity, rapidity, and availability. All known colorimetric determination methods of sulfated polysaccharides are based on interactions with cationic dyes such as methylene blue, dimethylmethylene blue [3–5], and toluidine blue [6, 7] in a solution. Being with high sensitivity, the described methods have essential insufficiencies such as necessity for creation of optimal conditions for formation of a complex of dye with sulfated polysaccharides. The presence of competing substances in a testing sample such as detergents, salts,

J. M. Lee · Z.-U. Shin · G. T. Mavlonov · T.-H. Yi (✉)
Graduate School of Biotechnology, Kyung Hee University, Yongin, Republic of Korea 446-701
e-mail: drhoo@khu.ac.kr

G. T. Mavlonov · I. Y. Abdurakhmonov
Center of Genomic Technologies, Institute of Genetics and Plant Experimental Biology, Academy Sciences of Uzbekistan, Tashkent, Uzbekistan 111226

acid, or alkali is a serious obstacle for these methods. Alternatively, "solid phase" methods of determination of the biopolymers, based on preliminary immobilization or fixation by precipitation of the target substances on the filter with subsequent removal of interfering substances and carrying out colorimetric reaction are well recommended in protein chemistry [8]. The development of a novel method for fucoidan determination and quantification using methylene blue staining from biological extracts, chromatography fractions, and biological liquids after immobilization of fucoidan on a filter paper was the purpose of this work. We also demonstrated the alternative approach using image processing software for fucoidan quantification without extraction of dye from stained paper spots of fucoidan–dye complex.

Materials and Methods

Materials

Methylene blue (MB) was purchased from Sigma (USA). Inorganic salts, organic solvents were from Samchun Chemical Co., LTD (Korea). For immobilization of fucoidan, a Whatman grade 1 chromatography paper (tickness, 0.18 mm; cat. no 3001–861) was used. Dried brown algae *Hizikia fusiformis* was purchased from a commercial supplier, Wando, Republic of Korea and it was certified by Professor You-Jin Jeon (School of Marine Biomedical Sciences, Jeju National University, Republic of Korea). A voucher specimen was deposited at Jeju National University.

Extraction and Purification of Fucoidan

Five hundred grams of air dried and powder of edible brown algae *H. fusiformis* was defatted by washing three times with 500 ml of acetone–water mixture (7:3). Residue was held at 40 °C in vacuum oven in an adequate time for removal of acetone and then was subjected for extraction with 2.5 L of water containing 0.1 M HCl as described [9]. Fucoidan was precipitated from the extract by adding an equal volume of 2 % CTAB. A pellet of fucoidan was collected by centrifugation and dissolved in 100 ml of 2 M NaCl in 0.1 M glycine–HCl pH3.0. Fucoidan solution was further dialyzed against 4 L of water and subsequently against 50 mM Tris–HCl pH7.5–7.8. For purification purposes, the dialyzed fucoidan solution was loaded into 2.5×15 cm Q-sepharose column equilibrated in 50 mM Tris–HCl pH7.8, 0.1 M NaCl. Column washed first with 500 ml equilibrating buffer and then 500 ml of 0.4 M NaCl. Both fractions containing neutral and mild acidic (low sulfated) polysaccharides were discarded. Here, we used the fucoidan from Q-sepharose column eluted with 150–200 ml of 3 M NaCl in water. The yield of high acidic fractions was measured with absorption at 210 nm or on refraction (Tunable absorption detectors 486 and RI 410, Waters, USA). Fucoidan fractions were dialyzed against water and lyophilized.

Solid-Phase Determination of Fucoidan with Dye Extraction from Stained Spot Methylene Blue-Fucoidan Complex

On a chromatography paper with 0.18 mm thickness (Whatman grade 1), the position of 1× 1 cm square spots were determined using graphite pencil. On the center of these squares, 2 μl of a standard solution containing various quantities of fucoidan in water or in a buffer solution (is stipulated) was dropped. When the quantity of loading fucoidan was in greater

than 2 μl, the next portions were dropped on the same point after drying of the last drop by hot air stream (fan). Finally, the paper with loaded fucoidan drops was dried using the fan, then was treated in mixture of methanol and acetone (6:4) for 2–3 min. Fucoidan spots on filter paper were visualized 10 min at room temperature in staining solution containing 0.1 %MB, 50 mM HCl in mixture of methanol/acetone/water (6:4:15). The excess of MB was removed by repeated washing of filter paper with mixed solution containing 5 % acetic acid, 6 % methanol, and 4 % acetone until an appropriate destained background appeared.

Filter paper squares containing stained fucoidan spots were cut according to preliminarily marked square lines and put into 1.5 ml Eppendorf centrifuge tubes with 0.5 ml of 70 % methanol containing 2 % SDS. MB from spots was extracted during 15 min at 50 °C. Of this extract, 0.2 ml was transferred into 96-well plate and absorbance was determined at 663 nm by means of Versamax microplate reader (Molecular Devices, USA). Data of triplicate experiments were analyzed using Microsoft Excel 2007 and graphics of absorbance correlation of fucoidan–MB complex at A_{663}nm was constructed, which corresponds to fucoidan content on a spot.

Solid-Phase Determination with Image Processing of Scanned MB-Stained Fucoidan Spots

The filter paper with MB-stained fucoidan spots was scanned and an image of appropriate format was processed with Quantity One® software (Bio-Rad, USA; version 4.6.3). MB-stained fucoidan spots were analyzed with "Volume analysis" option of above software. Results of triplicate experiments were analyzed using Microsoft Excel 2007 as indicated above and graphic of spot volume correlation in Quantity One® conventional units was constructed, which corresponds to fucoidan content on a spot.

Results and Discussion

The general property of natural sulfated polysaccharides like heparin, chondroitin, dermatan–sulfates (animals and human), carrageenans (red seaweed), and fucoidan (brown seaweed) is the presence of a negative charge in these molecules in wide range of pH [9] that allows them to form ionic complexes with organic cations [5]. The formation of similar complex with cationic dyes such as methylene blue [3–5] or toluidine blue [6, 7, 10] leads to batochrom shift in a visible spectrum in a solution. On the basis of this phenomenon, the colorimetric methods of detection for heparinoids were developed [4]. However, the ionic nature of above complexes with cationic dyes makes the colorimetric methods sensitive in a solution because of the presence of interfering cations and anions in the reaction media. In particular, the ionogenic components of biological liquids such as blood plasma, a lymphatic liquid, and urine can cause an essential interference. Because of these reasons, we have tested the possibility of colorimetric determinations of fucoidan from the dye-stained spots on filter paper. For the purpose of removal of possible complexes of cations from the samples containing fucoidan, which block the formation of a complex with dye and pollute the staining reagent, fucoidan on filter paper was washed with methanol/acetone (6:4) while stirring. The subsequent formation of a color complex had been reached by addition of 40 % mix of methanol/acetone (6:4). The addition of 50 mM HCl increased both specificity and sensitivity of fucoidan detection. It is shown in Fig. 1 that dependence of MB absorption on spot at 663 nm from fucoidan is linear in the range of 1–20 μg.

In order to test the specificity and sensitivity of described fucoidan determination method in biological environments, we studied possibility of analytic quantification in the presence

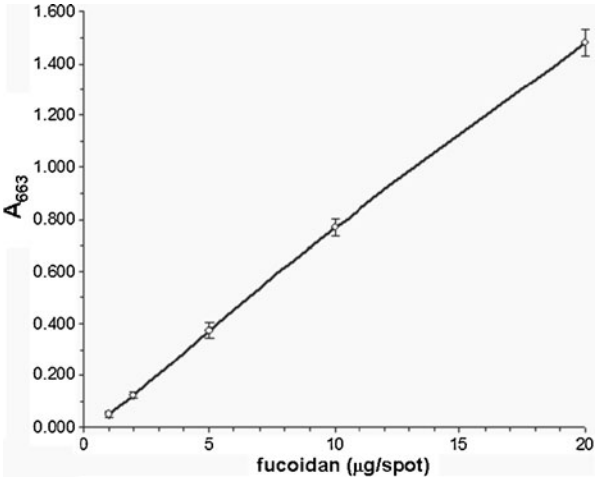

Fig. 1 A calibration curve of relations between A_{663} and fucoidan amount in spots

of potentially interfering substances, alginic acid (acidic polysaccharide of seaweeds containing glucuronic acid) and DNA, both these biopolymers can bind to MB at pH from slight acidic to highest alkali values. Two proteins, cow milk casein (acidic phosphoprotein) and RNase (a cationic protein) also were included as substances that possibly shielding the interaction of MB–fucoidan. We included sodium dodecyl sulfate as the most known dissociating agent of the complex formation. Results indicate (Table 1) that the detection of fucoidan does not deviate from an average of 10 μg with standard error of 17 % (SE=0.17).

In the following experiment, we tested the possibility of application of the given method in the biological liquids containing a high concentration of proteins and other biogenic substances (such as urea), capable to change charge of fucoidan. For imitation of blood plasma, we used a solution of human serum albumin in the phosphate-buffered saline. The solution at 1 mg/ml of urea containing ammonium acetate (2 mg/ml) was considered as imitation of the media of urine of animals. As demonstrated in Table 2, our solid-phase method correctly detected quantity of fucoidan in above-described conditions.

It is necessary to mention that variants of liquid–phase colorimetric quantification methodology of fucoidan and others sulfated polysaccharides described in the literature are sensitive to presence of the interfering substances tested in our work. Success of our method is provided by the removal of interfering substances from a surface of the fucoidan molecules on the filter in this solid–phase condition. Specifying the influence of hydrochloric acid (dissociation of fucoidans and ionic complexes, contribution to linkage with positively charged MB, increase of specificity of reaction by reduction of linkage MB with alginate, proteins and DNA at low pH) in quantification is an important step. At the same

Table 1 Determination of 10 μg fucoidan in presence of potential interfering substances

Interfering substances, amount (μg)	Average of determined fucoidan (μg)	SD	SE
Na-alginate, 10	10.147	0.25	0.15
DNA, 10	10.150	0.30	0.17
RNase, 10	9.820	0.30	0.17
SDS[a], 2 %	9.940	0.31	0.17
Control, water	10.000	0.15	0.09

SD standard derivation, *SE* standard error

[a]Fucoidan sample loaded to paper from solution containing 2 % of SDS

Table 2 Determination of 10 μg fucoidan in artificial biological fluids

Formulation	Determined fucoidan (μg)
Urea, 1 mg/ml	9.9±0.30
NaCl, 0.9%	
Ammonium acetate, 2 mg/ml	
HSA, 40 mg/ml	10.1±0.35
NaCl, 0.9 %	
K-phosphate 50 mM pH7.2	
Control (10 μg fucoidan in water)	9.9±0.30

HSA Human serum albumin

time, keeping immobilized fucoidan on a solid phase in the conditions of filter paper preparation is also important. This problem was dealt by replacement part of methanol with acetone in staining and washing solutions.

In scientific experiments, effective ways for data collection and its analysis are vital that require the use of precise techniques and computer software. We have tested possibility of using an image processing software for fucoidan quantification from a stained spot. We chose the Quantity One® software, which is one of known programs for processing digital images from stained thin layer chromatograms and electrophoregrams. Using digital quantification, the experimenter saves time, avoids additional errors, excludes MB dye extraction step from spot and subsequent colorimetric measurement, etc. As shown in Fig. 2, Quantity One® software allowed to obtain comprehensible data with linear curve from 1 to 10 μg per spot. The Quantity One method have linear curve comparable to spectrophotometric method, but within narrow range. This can be explained with the fact that in high concentration of fucoidan, the studied filter spot got deposited with multilayers of MB-stained complex, but computer scanner or camera registers only the spot surface intensity of the spot and bottom layers of the stained complex will stay "hidden". However, the presence of other sulfated biopolymers (heparin, chondroitin- and dermatan-sulfates, carrageenans), which give same

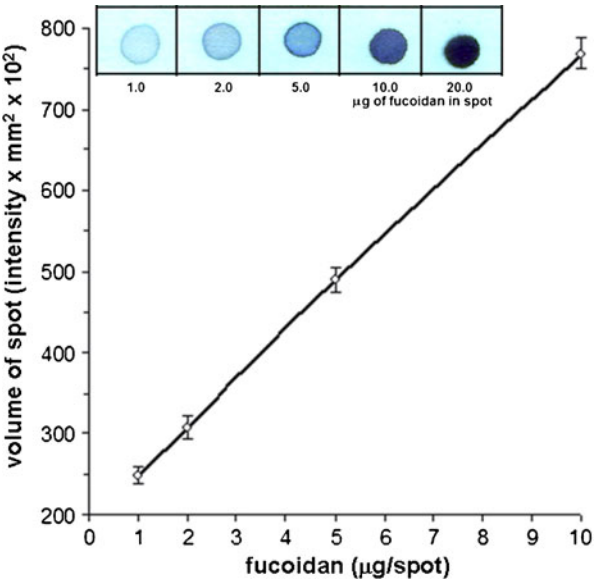

Fig. 2 The graph of dependence of optical density of the spot from the quantity of fucoidan measured by Quantity One®. Upper insertion is the fucoidan spot stained with methylene blue. The *graph* represents the quantity dependents in linear curve

complex with MB, results in reducing the specificity of fucoidan determination. In this case, the use of antibody is the only way for specific determination of fucoidan [11].

Conclusion

This method allows determination of fucoidan in biological samples and chromatographic fractions, containing salts, detergents, acidic or cationic biopolymers. Our method described herein effectively quantifies fucoidan and other sulfated polysaccharides determination without their specific discrimination.

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