Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Short communication

An immobilized perylene diimide derivative for fucoidan purification from a crude brown algae extract

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ARTICLE INFO

Keywords:
Fucoidan
Perylene diimide derivative
Beads
Purification

ABSTRACT

A polycationic perylene diimide derivative was successfully immobilized to capture polysulphated fucoidan from a crude extract of *F. vesiculosus*. At a slightly acidic pH, immobilized perylene diimide derivative is positively charged and can form electrostatically-driven aggregates with more than 80% of fucoidan in only 16 h, compared to 44 h in recently developed immobilized toluidine blue. Purified fucoidan was identified by FTIR, which demonstrated typical IR bands in comparison with the commercial product. Moreover, it showed improved purity than crude type by a factor of 1.4 after purification. Pharmacologically, it prolonged APTT and TT, revealing a potential heparin-like anticoagulant activity by interfering with intrinsic and common blood coagulation pathway. Automated downstream and scale-up processes were further performed with developing a FPLC protocol. The new technique proved to be a competitor to immobilized toluidine blue for production of a high-grade fucoidan.

1. Introduction

In the cell wall of brown algae, fucoidan functions as a cross linker between cell wall components cellulose and hemicellulose to promote cell integrity in addition to its potential moisture-regulating function during summer time and low tide periods [1]. Fucoidan is a bioactive water soluble, fucose-rich sulfated polymer with diverse and promising health benefits, such as anticoagulant [2], antiviral [3] and cytotoxic activities [4].

Interest in an industrial-scale and GMP-complied production of biologically-active polysaccharides including fucoidan has recently increased in pharmaceutical applications [5–7]. In addition, native fucoidan is urgently required to reveal its structure-activity relationship [8].

Even after pretreatment, extraction of fucoidan from either brown macroalgae or marine invertebrates is, in most cases, accompanied with contaminants (e.g., alginate, proteins, polyphenolics,….etc.) and needs a purification step later on [8]. Because a standardized purification technique has not yet been developed, several new techniques have been designed to try to reach the optimum fucoidan purity [9,10]. Previously, purification and fractionation of fucoidan has typically been executed by anion exchange chromatography as DEAE-cellulose [4,11,12], depending on anionic negative charge imparted by sulfated ester groups. Biological systems were also used, such as antithrombin III and heparin cofactor II, depending on their binding affinity to fucoidan in performing its anticoagulant function. Immobilization of these compounds on concanavalin A-Sepharose was interesting and successfully applied to purify fucoidan [13]. More recently, fucose-specific lectins were applied to purify fucoidan in a single step similarly to fucose-containing proteins [14,15]. Unfortunately, these techniques were expensive and time-consuming. Moreover, gel permeation chromatography should be afterwards performed to remove salts used in elution step and low molecular weight contaminants [8,10].

Similar to biological systems, immobilization of non-biological compounds with high affinity to fucoidan (such as thiazine dyes) was recently applied. The protocol was carried out in one-step utilizing the formation of a charge transfer complex between fucoidan and toluidine blue [16]. While this technique effectively captured fucoidan, it required a long time incubation (40–44 h) to perform its function in addition to providing a low yield [16,17].

A polycationic perylene diimide derivative (*N,N′*-Bis-(1-amino-4,9-diaza dodecyl)-1,7-di bromo perylene-3,4:9,10-tetracarboxylic acid diimide) is a fluorescent structurally-related compound to Heparin Red® (Fig. 1), the key component of commercial kits (Redprobes UG).

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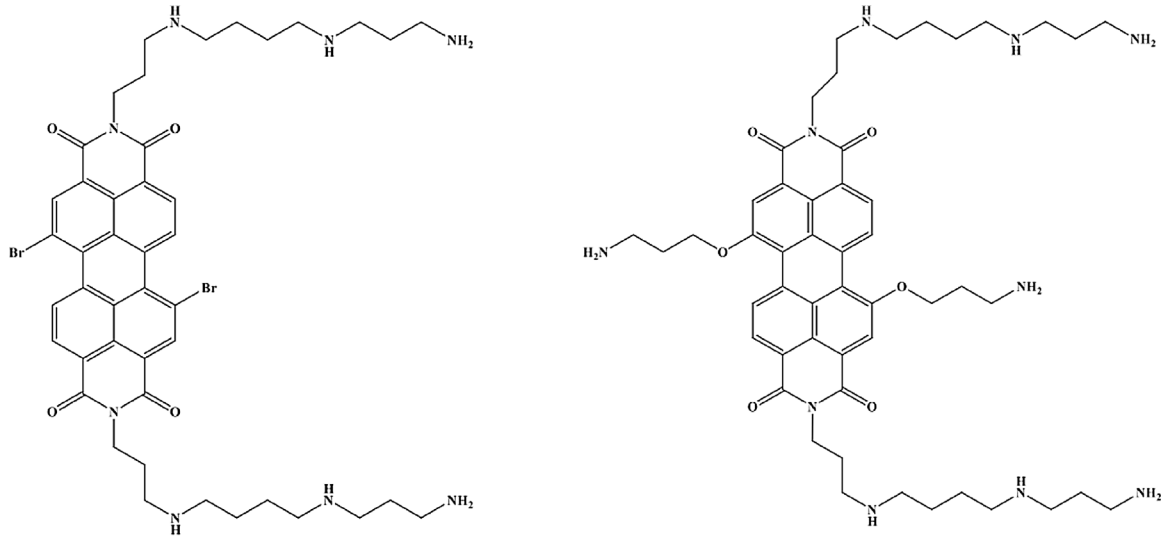
http://dx.doi.org/10.1016/j.procbio.2017.10.012
Received 5 July 2017; Received in revised form 28 September 2017; Accepted 16 October 2017



(A) (B)

Fig. 1. Molecular structures of A) perylene diimide derivative (*N,N′*-Bis-(1-amino-4,9-diaza dodecyl)-1,7-di bromo perylene-3,4:9,10-tetracarboxylic acid diimide. B) Heparin Red®. Perylene diimide derivative shows several primary and secondary amino groups, which are available for immobilization on amino-derivatized beads through a glutaraldehyde bridge as well as formation of an aggregate by electrostatic interaction of the protonated polyamine residues with polyanionic fucoidan in acidic or neutral pH.

Heparin Red® proved its effectiveness for the detection of heparin in plasma [18] and urine [19] and, more recently, for the quantification of fucoidan in spiked plasma [20] based on formation of electrostatically-driven aggregates with anionic sulfated heteropolysaccharides, such as heparin and fucoidan.

Chemically, perylene diimide derivative (PDD) might be a candidate to be carried on Sepabeads® EC-EA through a glutaraldehyde bridge, since it has structural similarity to thiazine dyes such as primary and secondary amino groups. Later on, derivatized protonated beads were applied for fucoidan purification from its crude extracts in slightly acidic medium.

In this research, perylene diimide derivative was covalently-immobilized on Sepabeads® EC-EA. The derivatized beads were evaluated afterwards as a capture tool for fucoidan from crude algal extract of the macroalgae *Fucus vesiculosus* and compared with previously developed immobilized toluidine blue.

2. Material and methods

2.1. Perylene diimide derivative synthesis

Synthesis of the red fluorescent perylene diimide derivative was performed as described previously by Szelke et al. [21]. In brief, 1,7-dibromoperylene-3,4,9,10-tetracarboxylic acid dianhydride was converted to the diimide derivative by reaction with tris-(*t*-Butoxycarbonyl) protected tetraamine spermine. After deprotection with trifluoroacetic acid, the product was isolated as a trifluoroacetate salt. The product was afterwards characterized by absorbance and fluorescence spectroscopy, mass spectrometry and analytical HPLC.

2.2. Perylene diimide derivative immobilization

As previously described [16], 2 mM aqueous solutions of perylene diimide derivative or toluidine blue were immobilized on Sepabeads® EC-EA, changing the beads from its white color to a red fluorescent one in case of perylene diimide derivative or cyan in immobilized of toluidine blue.

2.3. Fucoidan extraction from F. vesiculosus

F. vesiculosus was harvested from the North Sea at the region of south beaches of Wilhelmshaven (Germany, 53°31.236N, 8°13.849E). The algal biomass was dried and pretreated, and then fucoidan was extracted as previously described [17] followed by crude fucoidan storage at room temperature in a well-closed plastic container.

2.4. Fucoidan purification

2.4.1. Small scale process

A stock solution of crude fucoidan was prepared with 20 mM MES buffer at pH 6 with a concentration 2.5 mg/mL. Fucoidan was purified in 6 steps in 2 mL eppis: 1.5 mL of stock solution was incubated with 75 mg of derivatized beads with perylene diimide derivative and toluidine blue for 16 and 44 h, respectively, washed with water followed by 0.1 M NaCl (pH 2), eluted with 3 M NaCl before dialysis through 3.5 kDa MWCO membrane and finally, lyophilized to obtain a white, fluffy, purified fucoidan. The process was repeated for 3 cycles, and the % adsorbed and eluted fucoidan was calculated

2.4.2. Scale-up process

A FPLC protocol was developed to provide the same phases as a batch process. 3.2 g of derivatized beads with perylene diimide derivative were packed in a XK 16/20 column (72 × 16 mm, 4 cm^3). 1 mL of 50 mg/mL crude fucoidan in MES buffer was injected. In the elution step, the fraction collector started to collect the eluate in 2 mL portions. Fractions were then analyzed with toluidine blue assay. Fractions containing fucoidan were pooled for further steps of the purification protocol. Different flow rates were applied; 1 mL/min for column conditioning (15 mL) and washing steps (15 mL for water and 20 mL for 0.1 M NaCl), 0.5 mL/min (30 mL) in adsorption phase, and 2 mL/min (50 mL) in elution phase were applied.

2.5. Fucoidan analysis

Toluidine blue assay was performed to determine fucoidan content in supernatant and FPLC fractions during purification protocol [22]. Briefly, 10 µL of incubation supernatants were taken at 5, 60 and 960 min for immobilized perylene diimide derivative and 1080, 1440,