Algal Research 32 (2018) 54–59

Contents lists available at ScienceDirect

Algal Research

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

Fucoidanase inhibitory activity of phlorotannins from brown algae

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ARTICLE INFO

Keywords:
Fucoidanase
Inhibitors
Phlorotannins
Phlorethols
Fucophlorethols
Electrophoresis

ABSTRACT

The inhibitors of recombinant fucoidanase FFA2 from the marine bacterium *Formosa algae* KMM 3553^T were found among the metabolites of brown algae *Fucus evanescens* and *Costaria costata*. NMR spectroscopy and mass spectrometry analysis showed that high molecular weight phlorethols (degree of polymerization (DP) of 12–25 monomers) from *C. costata* (ICc) and fucophloretol (DP = 3) from *F. evanescens* (IFe) were inhibitors of fucoidanase. To inhibit one mole of FFA2 fucoidanase about two molecules of the ICc inhibitor or 7 molecules of the IFe inhibitor are needed. It has been suggested that the effectiveness of inhibition increases with the increasing molecular weight of phlorotannins. An irreversible type of inhibition was established.

1. Introduction

The search and study of enzyme inhibitors are important tasks for present-day enzymology. Inhibitors of enzymes are of great scientific importance and practical application in various fields, including biotechnology, pharmacology, and toxicology, and in the study of biochemical processes occurring in organisms. Inhibitors of high specificity are used to study the mechanisms of action of enzymes and to establish the structures of their active sites. Role of natural inhibitors in terrestrial plants is to protect the plants from the digestive enzymes of insects and herbivores—glycosidases, amylases and proteinases. A vital aspect of studying the role of enzyme inhibitors is to investigate the regulation of relationships among organisms. At present, the mechanisms of action of these inhibitors have been the most studied [1,2].

Brown algae are the main ingredient in the diets of marine organisms. They can be protected from being eaten by marine invertebrates or microorganisms by synthesizing, like terrestrial plants, inhibitors of alginate-lyase, laminarinases and fucoidanases – enzymes involved in the transformation of algal polysaccharides. Recently, fucoidans have become the object of increased attention and intensive research. The practical interest in these polysaccharides is explained by their low toxicity and various biological activities [3,4]. Fucoidans are a structurally diverse family of sulphated and sometimes acetylated homo- and heteropolysaccharides. An essential component of fucoidan molecules is residues of sulphated α-l-fucose. Fucoidanases are enzymes that catalyze the hydrolysis of fucoidans, and they are currently of particular interest. The role of these enzymes and their inhibitors is significant because all brown algae synthesize fucoidans. The total mass of these polysaccharides in nature is enormous and can thus be utilized in some way. An important trend in the use of fucoidanases and their inhibitors is the creation of drugs based on fucoidans. It is known that biological activity is often manifested by oligomeric fragments of polysaccharides [5,6]. Despite this, fucoidanases are poorly studied enzymes. This is due to several reasons: the lack of quantitative methods for determining the activity of fucoidanases and the use in studies as substrates of structurally uncharacterized or partially purified fucoidans. It is necessary to take into account the structural diversity of fucoidans, which assumes the presence in nature of a large number of fucoidan hydrolases of different specificity. There are few known sources of these enzymes. To date, fucoidanases have been isolated from the marine microorganisms *Vibrio* sp. N-5 [7] and “Fucobacter marina” SI-0098 (*Flavobacterium* sp. SA-0082) [8], the marine proteobacteria *Pseudoalteromonas citrea* KMM 3296, KMM 3297 and KMM 3298 [9], the marine invertebrates *Haliotus* sp. [10] and *Mizuhopecten yessoensis* (*Patinopecten yessoensis*) [11], and the marine urchin *Strongylocentrotus nudus* [12]. Additionally, fucoidanase FFA from the marine bacteria *Formosa algae* KMM 3553^T [13] and fucoidanases from the marine mollusks *Lambis* sp. [14], *Littorina kurila* [15] were isolated and investigated in our laboratory.

At the present time, there is only one our publication on the inhibitors of fucoidanases [16]. Discovered inhibitors, fucophloretol, belong to the class of phlorotannins – polyphenolic metabolites of brown algae. Fucophloretol was isolated from the brown alga *Fucus evanescens* and inhibited fucoidanase FFA from marine bacteria *F. algae*. It should be noted that algal phlorotannins are known as inhibitors of

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https://doi.org/10.1016/j.algal.2018.03.009
Received 11 November 2017; Received in revised form 9 February 2018; Accepted 15 March 2018

glycoside hydrolases, including α-fuco-, β-galacto- or β-mannosidases which present in the viscera of the marine mollusk [17].

In our studies, we continue to search for fucoidanase inhibitors among the metabolites of brown algae of Far Eastern seas. In the present study, we focus on the newly discovered fucoidanase inhibitor found in *Costaria costata* and continue the study structure and mechanism of action of the fucoidanase inhibitor from *F. evanescens*, discovered by us before [16]. The brown algae *F. evanescens* (Fucaceae) and *C. costata* (Laminariaceae) are widespread in the seas of the Far East of Russia. These algae are the most promising raw material for the industrial production of fucoidan and are objects for mariculture. Hence inhibitors of marine organism enzymes are not only of scientific but also practical interest. Additionally, it was interesting to compare the nature of substances inhibiting fucoidanase that are synthesized in algae belonging to different orders.

2. Materials and methods

2.1. Reagents and materials

Organic solvents, inorganic acids, and salts were commercial products (Laverna, Russia). Phloroglucinol (≥99%) (Sigma, St. Louis, MO, USA). The brown alga *Fucus evanescens* C. Ag (order Fucales) was collected in Iturup Island, Sea of Okhotsk, in August 2015. The brown alga *Costaria costata* (Turn.) Saund (order Laminariales) was collected in Peter the Great Bay, Sea of Japan in July 2016. Fucoidan was isolated from *F. evanescens* by the previously reported methods [18]. A construct encoding FFA2 (V37-A648) without the predicted signal sequence and C-terminal sorting domain was obtained, and the protein (FFA2) was produced in *E. coli* as described previously [19].

2.2. NMR spectra

The NMR spectra of fractions dissolved in MeOD were obtained on a Bruker Avance-III 500 HD spectrometer (Bruker, Karlsruhe, Germany) at an operating frequency of 500 MHz and 35 °C with tetramethylsilane as the internal standard.

2.3. Mass spectra

ESIMS spectra were recorded with an ESI Q-TOF mass-spectrometer Maxis impact Q-TOF (Bruker, Karlsruhe, Germany) with a direct infusion electrospray ionization source. Mass spectral data for ICc were obtained in the negative ion mode for a mass range of *m/z* 100 to *m/z* 1800. The capillary voltage was set to 4000 V and the drying gas temperature was 325 °C. The dried sample was dissolved in methanol (the concentration of the sample was approximately 0.5 mg/mL) and was introduced into the mass spectrometer at a flow rate of 5 μL/min using a syringe pump (KD Scientific, Holliston, MA, USA).

2.4. UV spectra

The ultraviolet (UV) absorbance spectrum from 200 nm to 400 nm for the sample solution (1 mg/mL in distilled water) was measured by a micro plate reader (BioTek Instruments Inc., Winooski, VT, USA).

2.5. Protein electrophoresis

SDS PAGE was performed according to Laemmli [20].

2.6. Isolation from C. costata of fractions with inhibitory activity

Freshly collected brown alga *C. costata* (7000 g) was rinsed with freshwater, cleaned to remove epiphytes, dried with filter paper, crushed and extracted with EtOH (96%, 10 L) for 30 days at room temperature. The extract was filtered, and an aliquot of 4.5 L was concentrated in vacuum to 1.3 L. The concentrate was extracted sequentially with hexane (3 × 500 mL), $CHCl_3$ (3 × 500 mL) and EtOAc (3 × 500 mL). The EtOAc extract was evaporated to dryness. The obtained residue (1050 mg) was separated by chromatography using a column of silica gel (1500 mm × 150 mm) into 12 fractions (1 − 12) that were eluted sequentially by C_6H_6 (fraction 1) and C_6H_6–EtOAc (stepwise gradient, 10:1–1:1 (fractions 2–5), EtOAc (fraction 6) (340 mg), $CHCl_3$ (fraction 7), $CHCl_3$–EtOH (stepwise gradient, 30:1–1:1 (fractions 8–11)), and EtOH (fraction 12). Fraction 6 was separated over Polychrome-1 (Reakhim, Russia) using H_2O–EtOH (stepwise gradient) into five fractions (6.1–6.5). Fraction 6.3, eluted by H_2O–EtOH (2.5:1, 111 mg), was separated over a column of silica gel 100C-18 (Sigma, St. Louis, MO, USA) using H_2O–EtOH (stepwise gradient 0–96 in 5% steps) to fraction 6.3.25, which was eluted by EtOH (25%, 7.8 mg). Column fractions were analyzed by TLC on Sorbfil plates (ZAO Sorbopolymer, Krasnodar, Russia) that were sprayed with $FeCl_3$ solution (50%), followed by heating to 70 °C. *Rf* values were determined using Me_2CO:C_6H_6:H_2O:HCO_2H (90:30:8:5 drops).

Isolation from *F. evanescens* of fractions with inhibitory activity was carried out in a similar manner with small changes. Namely, the fraction 11 was eluted from silica gel by $CHCl_3$-EtOH (1:1) for the purification on Polychrome 1 and further on C-18. Fraction 11.3.20 was eluted by 20% EtOH.

2.7. Determination of enzyme activity

Activity of fucoidanase FFA2 was determined by electrophoresis in a polyacrylamide gel (C-PAGE) according to the literature [13]. In brief, the reaction mixture containing 8 μL of enzyme solution (0.07 mg/mL) in 0.02 M Tris–HCl buffer pH 7.0 with 5 mM $CaCl_2$ and 10 μL of fucoidan from *F. evanescens*, solution (20 mg/mL) in the same buffer incubated at 34 °C for 10 min to 24 h. The reaction was stopped by heating at 80 °C for 5 min. The hydrolysis products were mixed with 5 μL of loading buffer containing a 20% solution of glycerol in water and 0.02% phenol red. The samples (5 μL) were electrophoresed through a 5% (w/v) stacking gel with 50 mM Tris–HCl buffer pH 6.8 and 27% (w/v) resolving polyacrylamide gel with 150 mM Tris–HCl buffer pH 8.8. The gel was 1 mm thick. Gel staining was performed with a solution containing 0.02% O-toluidine blue (Sigma, St. Louis, MO, USA) and 0.3% alcian blue in EtOH, AcOH and H2O with a volume ratio of 2:1:1. The gel images were visualized using a calibrated densitometer DS-800 (Bio-Rad, Hercules, CA, USA).

Fucoidanase activity was detected by the occurrence of charged oligosaccharide bands in the gel. Analysis of the optical density of reaction products (bands ladder) carried out using the Quantity One 4.6.7 software (Bio-Rad, Hercules, CA, USA). The residual activity of the fucoidanase was estimated from the integrated optical density of enzymatic hydrolysis products (ladders of bands) of the fucoidan. The optical density of reaction products without the addition of any compounds to the reaction mixture was chosen as 100% (Cr). All manipulations were carried out in triplicate.

2.8. Determination of effector capability of compounds extracted from C. costata on enzyme activity

2.8.1. Inhibition of fucoidanase FFA2

A solution of fucoidanase FFA2 (0.07 mg/mL, 8 μL) in Tris-HCl buffer (0.04 M, pH 7.0) containing $CaCl_2$ (5 mM) was treated with an effector solution (2 μL) at concentrations of 0.01–5 mg/mL, incubated for 20 min at room temperature (~22 °C), treated with a solution of fucoidan from *F. evanescens* (20 mg/mL) in the same buffer, and incubated for 10 min at 34 °C. An aliquot (5 μL) of the resulting mixture was analyzed by polyacrylamide gel electrophoresis (see above).

2.8.2. Determination of the type of enzyme inhibition

The type of inhibition was established by PAGE of native enzyme