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A comparative study of specificity of fucoidanases from marine microorganisms and invertebrates

M.I. Kusaykin[1,*], A.O. Chizhov[2], A.A. Grachev[2], S.A. Alekseeva[1], I. Yu Bakunina[1], O.I. Nedashkovskaya[1], V.V. Sova[1] & T.N. Zvyagintseva[1]
[1]*Pacific Institute of Bioorganic Chemistry, Far-East Branch of RAS, 690022, Vladivostok, 159 Prospect 100-letya Vladivostoku;* [2]*N.D. Zelinsky Institute of Organic Chemistry, RAS, 119991, Moscow, 47 Leninsky Prospect*

[*]Author for correspondence: e-mail: mik@piboc.dvo.ru; fax: (4232) 314-050

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Abstract

Specificities of actions of fucoidanases from the marine microorganism *Pseudoalteromonas citrea* KMM 3296 and the marine mollusk *Littorina kurila* were studied. The enzymes possess similar specificities and catalyze the cleavage of accessible α-(1→3)-fucoside bonds in fucoidans with highly sulfated α-(1→4; 1→3)-L-fucooligosaccharides. A high degree of sulfation of the fucose residues in fucoidans makes α-(1→3)-L-fucoside bonds inaccessible for the action of the studied enzymes. The maximum degree of cleavage of fucoidan was achieved by the fucoidanase from the marine bacterium *Pseudoalteromonas citrea* KMM 3296.

Introduction

Fucoidans, highly sulfated polysaccharides of brown algae, posses diverse biological activities. The most interesting are antitumor, anticoagulant, and antiviral activities, e.g., against HIV, hepatitis virus, and herpes virus (McClure et al., 1992; Nishino et al., 1991). For the last decade, the structure of these polysaccharides has been extensively studied. A close correlation between structural characteristics of fucoidans and the taxonomy of the corresponding brown algae was hypothesized: it is known that α-(1→3)-L-fucans are found in *Laminaria*, whereas species of *Fucus* genus mainly contain α-(1→3, 1→4)-L-fucans (Bilan et al., 2002). Structure/activity correlations for these polysaccharides are poorly studied. Usually fucoidans have a high d. p., so depolymerisation is needed for medicinal applications.

The enzymes degrading polysaccharides are widely used in structural studies, in studies of biological activities, and in preparation of drugs (Zvyagintseva et al., 1995). Fucoidanases are reported found only from marine organisms, and their activities are usually extremely low (Burtseva et al., 2000; Kusaykin et al., 2003; Bakunina et al., 2002). There are only a few studies on isolation and characterization of fucoidanases (Berteau & Mulloy, 2003). Information on the specificity of fucoidanases is scarce (the type of the glycosidic bond cleaved, and the influence of degree of sulfation of a substrate on the catalytic activity of these enzymes). Nevertheless, a fucoidanase from *Flavobacterium* sp. SA-0082 has been reported, and is already used for depolymerisation of fucoidan in the preparation of fucoidan-containing foods and beverages (Umeda et al., 1998). The most valuable sources of these enzymes from a technology standpoint, are still to be found.

The characterisitcs of enzymatic action of fucoidanases from a marine mollusc *Littorina kurila* and a marine bacterium *Pseudoalteromonas citrea* are presented in this paper.

Materials and methods

Analytical procedures

Neutral carbohydrates were quantified by the phenol-sulfuric acid method (Dubois et al., 1956); reducing

carbohydrates were determined according to Nelson (1944). Oligosaccharide composition was analyzed with a Jeol-JLC-6AH liquid chromatograph (Jeol, Japan) and a Bio Gel P-2 column (1 × 100 cm) eluted with 0.02 M acetate buffer, pH 5.4 at 16 mL/h^{-1}, orcinol- sulfuric acid assay. Monosaccharide composition was determined by HPLC with a LC-5001 carbohydrate analyzer (a Durrum DA-X8-11 column (385 × 3.2 mm) (Biotronik), bicinchoninate assay, and a C-R2 AX integrating system (Shimadzu)). The content of protein was determined by the method of Lowry (1951).

Substrates

Fucoidans from the brown algae *Laminaria cichorioides* and *Fucus evanescens* were isolated as described by Zvyagintseva et al. (1995).

Fucoidan from *F. evanescens* was purified as follows. To remove alginic acid, 100 mL of acetic acid was added to 300 mL of a solution of the fucoidan (50 mg mL^{-1}) and the precipitate formed was immediately centrifuged (9000 g, 10 min). The supernatant was neutralized with a solution of NaOH and the salt formed was removed by ultrafiltration at a 1 kDa cutoff (Sigma) using stepwise dilution. The resulting solution of fucoidan was applied to a column with DEAE-cellulose (Sigma) (20 × 30 cm) equilibrated with 0.01 M HCl and then eluted with a stepwise gradient of a NaCl solution (0.35, 0.5, 0.75, 1, 1.5, 2, and 3 M). The concentration of fucoidan was monitored by the phenol-sulfuric acid method (Dubois et al., 1956). The corresponding carbohydrate-containing fractions were pooled and dialyzed, then concentrated by ultrafiltration (1 kDa cutoff) and lyophilized.

Enzyme

Acidic (pH optimum at 5.4) and basic (pH optimum at 8.5) fucoidanases from a hepatopancreas of *L. kurila*, were isolated as described previously (Kusaykin et al., 2003). Fucoidanase from the bacterium *Pseudoalteromonas citrea* KMM 3296 was prepared as described by Bakunina et al. (2002).

Activities of enzymes

The activities of fucoidanases were determined by an increase of the amount of reducing sugars (Nelson et al., 1944). The incubated mixture contained 100 μL of the enzyme, 200 μL of a solution of fucoidan (4 μg mL^{-1}), and 200 μL of the corresponding buffer (0.05 M succinate buffer containing 0.2 M of NaCl, pH 5.4, or 0.05 M borate buffer, pH 8.5, or 0.01 M phosphate buffer, pH 7.2). The time of incubation did not exceed that needed to cleave 10% of the substrate in the incubated mixture. The amount of the enzyme which catalyzed the formation of 1 nmol of fucose for 1 h under conditions of determination was accepted as a unit of activity.

Preparation of products of enzymatic degradation of fucoidan

Fucoidanases from hepatopancreas of *L. kurila* were prepared as follows. Dry fucoidan (200 mg) was added to a solution of fucoidanases (20 mL, 10^{-2} units) in 0.05 M succinate buffer, pH 5.4 with 0.2 M of NaCl or in 0.02 M borate buffer, pH 8.5. After dissolution of the substrate, the mixture was incubated for 72 h at 37 °C. The reaction was stopped by boiling. High molecular weight products of the reaction were precipitated with ethanol (1:4, v/v). The fraction containing low molecular products of the reaction was evaporated to dryness *in vacuo* and then analyzed with an automatic liquid analyzer Jeol-JLC-6 AH. The product obtained using fucoidanase at pH 8.5 was separated by gel filtration on Bio Gel P-2, giving two fractions, P-1-L and P-2-L. Fraction P-1-L was subjected to ultrafiltration, 1 kDa cutoff. Non-dialyzable fraction (P-1-L) was analyzed.

Fucoidanase from bacterium KMM 3296 were prepared as follows. To a solution of fucoidanase (20 mL, 10^{-2} units) in 0.05 M phosphate buffer, pH 7.2, 200 mg of dry fucoidan (*F. evanescens*) was added. After dissolution of the substrate, the mixture was incubated for 7 days at 37 °C under sterile conditions. The reaction was stopped by boiling. The resulting products were separated on DEAE-cellulose (1 × 15 cm), the carbohydrate-containing fractions were desalted on Sephadex G-10 (1 × 50 cm), evaporated to dryness *in vacuo* and analyzed an automatic liquid analyzer Jeol-JLC-6 AH on a Bio Gel P-2 column (1 × 100 cm).

Desulfation of fucoidans and the product of their enzymatic cleavage

Fucoidan (50–100 mg) was transformed to a pyridinium form (Zvyagintseva et al, 2003) and dissolved in 18 mL of DMSO and 2 mL of pyridine by stirring then heating for 10 h at 100 °C. The solution was poured into water and DMSO was removed by ultrafiltration on a Millipore membrane with 1000 Da