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

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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

cutoff. The aqueous solution was concentrated and lyophilyzed.

Methylation of fucoidans and preparation of partially methylated polyol acetates was carried out as reported previously (Chizhov et al., 1999 and references therein). GLC-MS analysis of partially methylated polyol acetates was done with a Finnigan MAT ITD-700 (ion trap detector) mass spectrometer coupled with a Carlo Erba series 4200 gas chromatograph (capillary column column Ultra-1, Hewlett Packard, crosslinked polymethylsiloxane, 25 m length, 0.25 mm internal diameter, 0.33 μ liquid film thickness). Temperature program: isotherm 150 °C (1 min), then ramp 5 °C/min to 280 °C. Helium was used as a carrier gas. The component ratios were approximated by total ion current (TIC).

Results and discussion

It was shown previously that the marine bacterium *Pseudoalteromonas citrea* KMM* 3296 and the marine mollusk *Littorina kurila* have significant activities of fucoidanases (Burtseva et al., 2000; Bakunina et al., 2002). Fucoidanases from these resources have been partially purified and their properties studied (Kusaykin et al., 2003). Here we present the results of a comparative study of the specificity of three fucoidanases; i.e., basic (pH optimum at 8.5) and acidic (pH optimum at 5.4) from hepatopancreas of *L. kurila* and fucoidanases from *P. citrea* KMM 3296 (pH optimum at 7.2). Notably, the starting level of fucoidanase activity in the microbial source was one order of magnitude higher than in hepatopancreas of *L. kurila.*

Fucoidans from the brown algae *Fucus evanescens* and *Laminaria cichorioides* were used as substrates. Fucoidan from *F. evanescens* contained Fucose (95% on neutral carbohydrates content), Xylose (2.8%), Mannose (0.2%), Glucose (2%), and the molar ratio fucose:SO_4^{2-} was equal to 1:0.43. For the desulfated sample of this fucoidan, methylation analysis gave the ratio of acetates as 2,3,4-tri-O-methyl-:2,3-di-O-methyl-:2,4-di-O-methyl:2-O-methyl:3+4-O-methylfucitols as follows: 23:11:39:9:18. So, fucoidan from *F. evanescens* used as a substrate is a partially sulfated α-(1→3; 1→4)-L-fucan (linkage ratio 1→3:1→4 is 3.5:1). This fucoidan fraction substantially differs from that isolated previously (Bilan et al., 2002) from *F. evanescens*, which is a linear polymer with alternating (1→3)- and (1→4)-linked fucose residues sulfated mainly by C-2 and partially acetylated by other hydroxy groups. Fucoidan from *L. cichorioides* is almost totally sulfated α-(1→3)-L-fucan (Zvyagintseva et al., 2003).

The characteristics of the products of exhaustive enzymolysis of substrates (fucoidans from *L. cichorioides* and *F. evanescens*) are given in Table 1. The maximum degree of cleavage was obtained for fucoidan from *F. evanescens* by fucoidanase from *P. citrea* KMM 3296. When using acidic fucoidanase from *L. kurila* to cleave fucoidan from *L. cichorioides,* formation of low-molecular products is three times lower than for fucoidan from *F. evanescens* (Table 1). This fact may be explained by the greater accessibility of O-glycosidic bonds in low sulfated fucoidan from *F. evanescens* in comparison to highly sulfated fucoidan from *L. cichorioides*.

The action of the basic form of fucoidanase from *L. kurila* on fucoidan from *F. evanescens*, yielded three times more low-molecular products than the action

Table 1. The products of enzymatic cleavage of fucoidans by fucoidanases

		Characteristics			
Enzyme (pH-optimum)	Substrate (m wt kDa)	HMP, yields[a], %	n[b]	LMP, yields[c], %	n
Acidic fucoidanase (5.4)	Fucoidan from *F. evanescens*, 60	85	$n > 7$	15	$7 > n > 2$
	Fucoidan from *L. cichorioides*, 20	95	$n > 7$	5	$7 > n > 2$
Basic fucoidanase (8.5)	Fucoidan from *F. evanescens*, 60	55	$n > 7$	45	$7 > n > 2$
Fucoidanase from *P. citrea* KMM 3296 (7.2)	Fucoidan from *F. evanescens*, 60	30	$n > 7$	70	$5 > n > 2$

[a]HMP: highly molecular products obtained by precipitation with 80% aqueous ethanol (in % of total amount of products).
[b]n: degree of polymerization.
[c]LMP: low molecular products.

Table 2. The characteristics of low-molecular products of enzymatic cleavage of fucoidan from *F. evanescens* by action of fucoidanase from hepatopancreas of *L. kurila* and *P. citrea* KMM 3296

A source of enzyme	Products	% from Yield, the starting substrate	M. wt., kDa or n[a]	Carbohydrate composition, %						Molar ratio Fuc:SO_4^{2-}
				Fuc	Gal	Xyl	Rha	Glc	Man	
Pseudoalteromonas	P-1-Ps	26	$5 \geq n \geq 2$	96	4	0	0	0	0	1:0.31
citrea KMM 3296	P-2-Ps	8	2–3	97.2	0.4	2.1	0.3	0	0	1:0.53
Hepatopancreas	P-1-L	30	3–10	92	1	1.8	0	2.5	3.7	1:0.59
Littorina kurila,	P-2-L	8	$7 \geq n \geq 2$	50	0	0	0	50	0	0
	P-1-1-L	17	3–10	92	1	1.8	0	2.5	3.7	1:0.59

[a] n: degree of polymerization of products.

of the acidic form of the fucoidanase on the same fucoidan.

To study enzymatic transformation in detail, fucoidan from *F. evanescens* as a substrate and two enzymes (basic fucoidanase from *L. kurila* and fucoidanase from *P. citrea* KMM 3296) were chosen. The products obtained from the action of fucoidanases from *P. citrea* KMM 3296 on this fucoidan, as separated by ion exchange chromatography on DEAE-cellulose, yielded the two fractions (P-1-Ps and P-2-Ps) shown in Table 2. Acid hydrolysis of the products showed that they consist mainly of fucose. All fractions obtained by transformation of fucoidan with the microbial enzyme had sulfate groups and the total content of sulfate remained practically constant in comparison to starting fucoidan.

Gel chromatography on Bio Gel P-2 of the fraction P-1-Ps showed that it is a mixture of di-, tri-, tetra-, and pentafucooligosaccharides. This fraction (yield 26%, Table 2) has 30% by wt. of sulfate, which corresponds to sulfation one of two hydroxyls in the fucose residues. Solvolytic desulfation followed by methylation analysis of the fraction P-1-Ps gave the ratio of acetates of 2,3,4-tri-O-methyl-:2,3-di-O-methyl-:2,4-di-O-methyl:2-O-methyl-:3- and 4-O-methylfucitols equal to 11:50:26:13: none. Thus, the ratio of (1→4)- and (1→3)-linked fucosyl residues changed from 3.5 in the starting fucoidan to 0.5 in P-1-Ps, which demonstrates the predominant cleavage of α-(1→3)-glycosidic bonds by the fucoidanase from *P. citrea* KMM 3296.

The products formed by the action of basic fucoidanase from *L. kurila* (Table 2) were separated as follows. High molecular weight products of enzymatic cleavage of fucoidan were precipitated with 80% aqueous ethanol. Low molecular weight products remaining in the solution were separated on Bio Gel P-2, giving two fractions, P-1-L and P-2-L. Fraction P-1-L was subjected to ultrafiltration on a membrane with 1 kDa cutoff. Results of the analysis of the non-dialyzable fraction (P-1-1-L) are given in Table 2. Total acid hydrolysis gave Fucose (92%), Xylose (1,8%), Mannose (3,71%), and Glucose (2,5%); sulfate content was 40% by wt. in P-1-1-L. Solvolytic desulfation followed by methylation analysis gave the ratio of acetates 2,3,4-tri-O-methyl-:2,3-di-O-methyl-:2,4-di-O-methyl:2-O-methyl:3- and 4-O-methylfucitols equal to 10:41:31:11:7. The data show that the ratio of (1→4)- and (1→3)-linked fucosyl residues changed from 3.5 to 0.75 in P-1-1-L, which also demonstrates the predominant cleavage of α-(1→3)-glycosidic bonds by the fucoidanase from *L. kurila*. In the ^{13}C NMR spectrum of P-1-1-L, the most intense signals at 96.5 (C1), 69.1 (C2), 70.2 (C3), 81.1 (C4), and 68.8 ppm (C5) are preliminarily assigned to the fragment →4)-α-L-FucP-(1→ and weaker signals at 97.3 (C1), 67.5 (C2), 76.6 (C3), 69,8 (C4), and 67.7 ppm (C5) may be assigned to the →3)-α-L-FucP-(1→ link; in addition, the following signals were putatively assigned to the →3, 4)-α-L-FucP-(1→ fragment ((C-3, C-4)-branching points): 101.5 (C1), 68.4 (C2), 77.2 (C3), 70.2 (C4), and 67.7 ppm (C5).

Conclusions

The fucoidanases from the marine mollusk *L. kurila* and the marine bacterium *P. citrea* KMM 3296 have a similar specificity: they catalyze the predominant cleavage of α-(1→3)-glycosidic bonds between fucose residues in the polysaccharide. In contrast to fucoidanase from *L. kurila*, the bacterial fucoidanase cleaves fucoidan forming mainly di-, tri-, tetra-, and pentafucooligosaccharides, whereas the action of the basic form of fucoidanase from *L. kurila* yields higher molecular weight products of 3–10 kDa (Table 2).

Probably, these differences are related to structural peculiarities of active centers of enzymes and the mechanism of action of the enzymes on the polymer substrate.

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