

Effect of Enzyme Preparation from the Marine Mollusk *Littorina kurila* on Fucoidan from the Brown Alga *Fucus distichus*

M. I. Bilan¹, M. I. Kusaykin², A. A. Grachev¹, E. A. Tsvetkova¹,
T. N. Zvyagintseva², N. E. Nifantiev¹, and A. I. Usov^{1*}

¹Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninskii pr. 47,
119991 Moscow, Russia; fax: (7-095) 135-5328; E-mail: usov@ioc.ac.ru

²Pacific Institute of Bioorganic Chemistry, Far-Eastern Branch, Russian Academy of Sciences,
pr. 100-letiya Vladivostoka 159, 690022 Vladivostok, Russia

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Abstract—A fucoidanase preparation from the marine mollusk *Littorina kurila* cleaved some glycosidic bonds in fucoidan from the brown alga *Fucus distichus*, but neither fucose nor lower oligosaccharides were produced. The main product isolated from the incubation mixture was a polysaccharide built up of disaccharide repeating units $\rightarrow 3$ - α -L-Fucp-(2,4-di-SO₃⁻)-(1 \rightarrow 4)- α -L-Fucp-(2SO₃⁻)-(1 \rightarrow , the structure coinciding with the idealized formula proposed for the initial substance. A polymer fraction with the same carbohydrate chain but sulfated only at positions 2 and nonstoichiometrically acetylated at positions 3 and 4 of fucose residues was isolated as a minor component. It is suggested that the native polysaccharide should contain small amounts of non-sulfated and non-acetylated fucose residues, and only their glycosidic bonds are cleaved by the enzyme. The enzymatic hydrolysis showed that irregular regions of the native polysaccharide containing acetylated and partially sulfated repeating units were assembled in blocks.

Key words: brown algae, fucoidan, *Fucus distichus*, fucoidanase, *Littorina kurila*, marine mollusks

Fucoidans are polysaccharides built up largely of sulfated α -L-fucose residues. These biopolymers are mainly synthesized by brown algae [1, 2] and are also found in marine echinoderms [3]. Fucoidans display diverse biological activities [4] that seem to be due to their ability to react selectively with some proteins and to modify specifically the cell surface. For a long time biological features of fucoidans were thought to depend only on the high extent of sulfation of these polysaccharides [5], but fine details of their structure have been recently found to be also important [4]. However, in every case the establishment of chemical structure of fucoidans is rather labor consuming. First of all, molecules of great majority of algal fucoidans lack visible signs of regularity. Structural analysis of these biopolymers is still more complicated also because they are highly sulfated and contain minor components (xylose, galactose, uronic acids, acetyl groups). Therefore, only a few algal fucoidans have more or less reliably established structure [6-11] and, consequently, no specific biological activity can be associated with particular structures of fucoidan molecules.

Enzymes catalyzing partial cleavage of these polysaccharides, namely fucoidanase and sulfatase, would be useful tools for investigating structural features of fucoidans. Such enzymes have been found mainly in marine bacteria [12] and invertebrates—echinoderms [13] and mollusks [14]—and the effect of these enzymes on algal fucoidans have been studied in some works [12, 14-17]. These studies are difficult because of rather low content of fucoidanases in both bacteria and mollusks (as compared, for example, to glucanases); moreover, purification of these enzymes is virtually always associated with a significant loss of their activities [15]. As a result, researchers often work only with partially purified enzyme preparations [15] or even with complexes of polysaccharide hydrolases [16]. Moreover, unfractionated fucoidans with unknown structure are usually used as substrates in studies on specificities of fucoidan-degrading enzymes. Therefore, although the structure of low-molecular-weight fragments of fucoidans (fucooligosaccharides) has been studied in detail elsewhere [16], it is not always possible to compare structures of oligosaccharides and the initial native polymer, and thus determine the specificity of the enzyme effect. Nevertheless, two

* To whom correspondence should be addressed.

publications on this subject seem to deserve attention. The work [17] dealt with a regioselective sulfatase isolated from the marine mollusk *Pecten maximus*, which hydrolyzed sulfate groups only at positions 2 of fucopyranosides. In work [18] treatment of fucoidan from *Cladosiphon okamuranus* by the enzyme complex from the marine bacterium *Fucophilus fucoidanolyticus* resulted in a set of oligosaccharides with structures suggesting the presence of pentasaccharide repeating units in the initial polysaccharide. Fucoidanase in this complex cleaved only glycosidic bonds of fucose residues substituted by a glucuronic acid residue at position 2.

The present work is an extension of studies on properties of enzymes from the marine gastropod *Littorina kurila* that catalyze transformation of fucoidans. The hepatopancreas of *L. kurila* was earlier shown [19] to contain an enzyme, which displayed the fucoidanase activity to fucoidans from the brown algae *Fucus evanescens* and *Laminaria cichorioides*. Fucoidanase was isolated and purified from α -fucosidase and arylsulfatase. In the present work, the fucoidan fraction from *F. distichus* was used as a substrate for fucoidanase. This fraction is a polysaccharide with high regularity that is mainly built up of identical repeating disaccharide units [10], and it differs from *Laminaria cichorioides* fucoidan in the structure of the backbone and from *F. evanescens* fucoidan in distribution of sulfate groups and by lower extent of acetylation.

MATERIALS AND METHODS

Fucoidanase was isolated from the hepatopancreas of *L. kurila* and purified as described in [19]. A highly sulfated fraction of fucoidan (F_4) from *F. distichus* was prepared as described in [10]. The protein was quantified by the Lowry's method [20] with BSA as a standard. Neutral monosaccharides were quantified by GLC after hydrolysis of polysaccharides (2 M CF_3COOH , 8 h at 100°C) as polyol acetates [21] or aldonitrile acetates [22], with *myo*-inositol acetate as an internal standard. GLC was performed using a Hewlett-Packard 5890A chromatograph equipped with an HP Ultra-2 capillary column, flame-ionization detector, and an HP 3393A integrator, at temperature programmed from 175 to 290°C at the rate of 10°C/min. Sulfate was determined turbidimetrically as BaSO_4 after hydrolysis of polysaccharides in 1 M HCl [23].

Determination of fucoidanase activity. The activity was determined as described in [19] by increase in the concentration of reducing sugars registered by the Nelson's method [24]. The incubation medium contained 100 μl of the enzyme preparation, 200 μl of fucoidan F_4 solution (4 mg/ml), and 200 μl of 0.05 M acetate buffer (pH 5.4). The incubation time was 5 h. The enzyme amount that increased the reducing capacity of

the incubation medium equivalently to production of 1 μmol fucose per 1 h was taken as the enzyme activity unit.

Enzymatic cleavage of fucoidan. A solution (25 ml) of fucoidanase (activity of 0.23 U/ml) in 0.05 M acetate buffer (pH 5.4) containing 0.2 M NaCl was supplemented with 245 mg fucoidan. On dissolving the substrate, the medium was incubated for 21 h at 37°C, supplemented additionally with 5 ml of the enzyme solution, and incubated further under the same conditions for 24 h, with sampling of 0.1 ml aliquots at chosen time intervals and determining in them the quantity of reducing sugars as described in [24]. Then the reaction was stopped by heating on a boiling water bath for 10 min. The resulting precipitate was separated by centrifugation and discarded, and to the supernatant four volumes of ethanol were added. The precipitate (high-molecular-weight reaction products (HMP)) was separated by centrifugation, washed with ethanol, dissolved in a minimal volume of water, and lyophilized (the yield was 210 mg), whereas the supernatant (low-molecular-weight reaction products (LMP) and salts from the buffer) was evaporated to dryness in vacuum (the yield was 140 mg).

The enzyme solution without the polysaccharide and the polysaccharide solution without the enzyme were incubated similarly as control reactions.

Gel-permeation chromatography. Columns with TSK HW-40(S) gel (Toyopearl, Japan) (2.7×76 cm, separation range in molecular weight of dextrans 0.1-7 kD) and with TSK HW-65(S) gel (Toyopearl) (2.7×60 cm, separation range in molecular weight of dextrans 10-1000 kD) were used. The columns were eluted with water at the rate of 2 ml/min and the eluted material was detected with a differential refractometer (Knauer, Germany).

Electrophoresis. Electrophoresis was performed in 0.6% agarose gel ($120 \times 110 \times 2$ mm) in 50 mM 1,3-diaminopropane acetate buffer (pH 9.0) at 110 V for 1 h. The gel was fixed, dried, and stained as described in [3].

NMR spectroscopy. NMR spectra were obtained using a DRX-500 spectrometer (Bruker, Germany) after the samples had been once or twice lyophilized from D_2O and dissolved in 99.96% D_2O . The spectra were calibrated with 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid, sodium salt (internal standard, δ_{H} 0).

RESULTS AND DISCUSSION

The highly sulfated fraction of fucoidan (F_4) isolated from *F. distichus* and consisting virtually only of fucose and sulfate was deliberately chosen as the substrate for the fucoidanase under study. It is the only known example of a highly regular algal fucoidan. The polysaccharide molecules include alternating residues of 3-linked α -L-fucopyranose sulfated at positions 2 and 4 and of 4-linked α -L-fucopyranose sulfated only at position 2. In other

words, the polysaccharide molecules contain the disaccharide repeating unit $\rightarrow 3)-\alpha\text{-L-Fucp}-(2,4\text{-di-SO}_3^-)-(1\rightarrow 4)-\alpha\text{-L-Fucp}-(2\text{SO}_3^-)-(1\rightarrow$. This regularity is only slightly masked by the presence in the polysaccharide of a small amount of O-acetyl groups and also by incomplete sulfation of some repeating units: the molar ratio of L-fucose, sulfate, and acetyl groups is 1 : 1.21 : 0.08. Nevertheless, these disorders of the regularity did not affect the quality of NMR spectra, and we were able to establish the structure of a native algal fucoidan without degradative approaches by NMR spectroscopy [10].

An enzyme for cleaving fucoidan was prepared from hepatopancreas of the marine gastropod *L. kurila*. According to [19] this source has two fucoidanase forms: an acidic one with pH optimum of 5.4 and an alkaline one with pH optimum of 8.5. The specific effect of alkaline fucoidanase on algal fucoidans was shown earlier [25], the specificity of the acidic form not being studied. In the present work, we studied the effect of acidic fucoidanase on fucoidan with known structure to investigate the enzyme specificity and obtain additional information about structural features of the fucoidan used as the substrate.

The enzymatic hydrolysis of fucoidan was followed by quantitative determination of reducing sugars in the incubation medium. Although the reducing ability of the fucoidanase preparation was initially high, it increased only in the presence of both the enzyme and the polysaccharide (Fig. 1). In two control experiments (incubation under the same conditions either of the enzyme or the polysaccharide), the reducing ability of the solutions remained virtually unchanged. These findings suggested that the fucoidanase under study hydrolyzed some glycosidic bonds in fucoidan molecules from *F. distichus*. On termination of the enzymatic hydrolysis and removal of

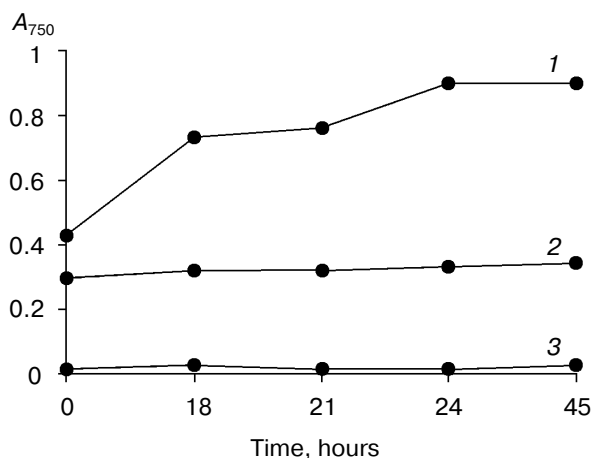


Fig. 1. Time-dependent changes in the reducing ability of incubation mixtures of fucoidan with the enzyme (1), the enzyme without fucoidan (2), and fucoidan without the enzyme (3).

Table 1. Composition of the initial fucoidan F_4 and polysaccharide fractions resulting from enzymatic hydrolysis

Fraction	Neutral monosaccharides, %			SO ₃ Na, %
	Fuc	Xyl	Gal	
F_4	40.8	0.8	0.8	34.8
HMP	40.9	1.2	1.9	35.3
I-1	44.0	0.5	0.8	29.9

the protein, high-molecular-weight reaction products (HMP) were separated from low-molecular-weight products (LMP) by precipitation with ethanol. A similar treatment of the polysaccharide solution incubated as described but without the enzyme did not result in separation of a low-molecular-weight fraction.

The composition of the high-molecular-weight fraction of the enzymatic hydrolysis products was virtually the same as that of native fucoidan F_4 (Table 1). ¹H- (see further Fig. 3) and ¹³C-NMR spectra of this fraction were also identical to similar spectra of F_4 . Thus, the HMP fraction was a part of the initial fucoidan not noticeably changed by the enzyme.

Attempting to reveal among enzymatic hydrolysis products short fucooligosaccharides (with 2-20 monosaccharide residues in the chain), the LMP fraction was studied by gel-permeation chromatography on TSK-HW-40(S) gel (separation range in molecular weight of dextrans was 0.1-7 kD). Only fraction I resulting by chromatography consisted of carbohydrates (by NMR data) and was eluted from the column with the void volume (i.e., its molecular weight was ≥ 7 kD). Thus, no oligosaccharides and monosaccharides were found among the products of enzymatic hydrolysis.

By chromatography on a column with TSK-HW-65 gel (separation range in molecular weight was 10-1000 kD), fraction I was divided into two fractions, of which only the I-1 fraction consisted of carbohydrates. The yield of the I-1 fraction was 6.1% (15 mg) of the initial fucoidan F_4 . The elution profile of the I-1 fraction on TSK-HW-65 was slightly different from that of the initial fucoidan. The difference in molecular weights of the I-1 fraction and native fucoidan was confirmed upon their reduction by sodium borohydride, subsequent hydrolysis, and comparison of fucose and fucitol molar ratios in the hydrolyzates. For the I-1 fraction this ratio was 64 : 1, which corresponded to the average length of the chain of 65 monosaccharide residues. The similar ratio for the native fucoidan was 424 : 1, i.e., the average length of the chain was 425 fucose residues. The difference in the molecular weight and/or charge density between the I-1

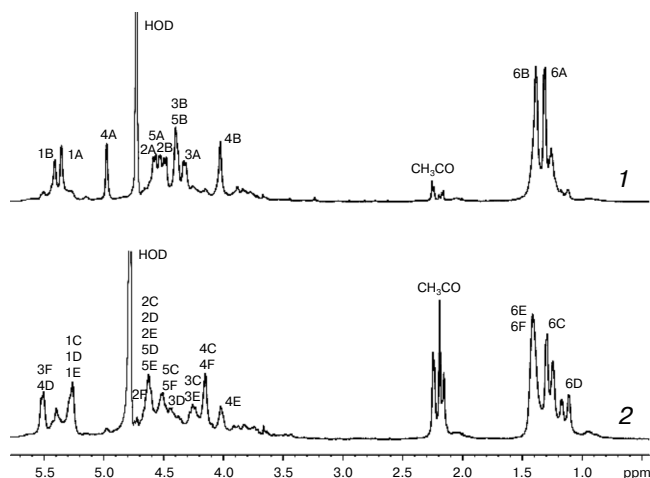


Fig. 2. Electrophoresis in 0.6% agarose gel of native fucoidan F₄ (1) and the I-1 fraction (2).

fraction and the native fucoidan was also shown by electrophoresis in agarose gel (Fig. 2). Table 1 shows that the I-1 fraction is different from the initial fucoidan F₄ also by lower content of sulfate groups (the molar ratio of fucose and sulfate was 1 : 1).

Structure of the I-1 fraction prepared by enzymatic hydrolysis was then studied by NMR spectroscopy. The ¹³C-NMR spectrum of this fraction was more complicated than a similar spectrum of native fucoidan F₄. At least three intense signals in the anomeric carbon resonance region (94-100 ppm) and also signals in the high field region (16.7 and 16.8 ppm) in the spectrum were typical for α-fucopyranosides. Four intense signals of carbons of O-acetyl groups (two in the high-field region at 20-22 ppm and two in the resonance region of carboxyl groups at 174-175 ppm) were distinctive for the ¹³C-NMR spectrum of the I-1 fraction as compared to the corresponding spectrum of native fucoidan F₄, where these signals were faintly visible. The ¹H-NMR spectrum of the I-1 fraction contained a number of intense signals in the anomeric (5.2-5.5 ppm) and high-field (1.0-1.4 ppm) regions. The signals at 2.2 ppm indicated the presence of O-acetyl groups in the polymer. The molar ratio of fucose and acetate calculated from integral intensities of signals of the appropriate protons was 1 : 0.4, i.e., the I-1 fraction contained fivefold more O-acetyl groups than native fucoidan (Fig. 3).

The resolution of both proton and carbon spectra of the I-1 fraction was sufficient for application of different variants of two-dimensional NMR spectroscopy used for assignment of the spectral signals. Heteronuclear two-dimensional correlation ¹H,¹³C HSQC revealed three cross-peaks in the anomeric region. Analysis of homonuclear ¹H,¹H two-dimensional spectra COSY and TOCSY allowed us to determine chemical shifts of all proton sig-

nals of the fucopyranose residues. Then signals in the ¹³C-NMR spectrum were assigned using the HSQC correlation (Table 2). Combined analysis of chemical shifts in the proton and carbon spectra revealed several types of fucopyranose 2-sulfate residues that were different in positions of glycosylation and O-acetyl groups. Analysis of 2D ROESY spectrum data (NOE) revealed that the I-1 fraction had a disaccharide repeating unit built up of 1→3- and 1→4-linked α-L-fucopyranose 2-sulfate residues, and some of the 4-linked fucose residues contained O-acetyl groups in position 3, while some of 3-linked residues were acetylated at position 4. The presence of three cross-peaks in the anomeric region of the HSQC spectrum was shown to be caused by differences in chemical shifts of anomeric protons and carbons of acetylated and non-acetylated 4-linked fucopyranose 2-sulfate residues.

Thus, treatment of fucoidan F₄ from *F. distichus* with the enzyme from *L. kurila* resulted only in a slight cleavage of the substrate. As shown by NMR spectroscopy, the major part of the substance remained unchanged; consequently, the enzyme preparation was unable to cleave the sequence consisting of alternating residues of 3-linked α-L-fucopyranose 2,4-disulfate and 4-linked α-L-fucopyranose 2-sulfate. The I-1 fraction prepared with a small yield was a polysaccharide built up of alternating 3- and 4-linked α-L-fucopyranose 2-sulfate residues partially acetylated at the remaining positions. Note, that the control treatment of the polysaccharide in the absence of the enzyme and the subsequent fractionation did not produce

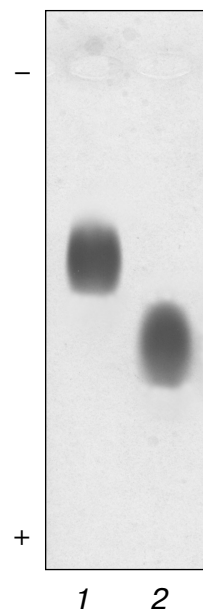
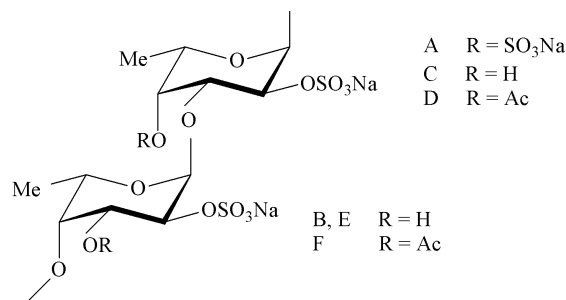


Fig. 3. ¹H-NMR spectra of the HMP fraction (1) and of the I-1 fraction (2). The letters and figures indicate the corresponding protons in residues A, B, C, D, E, and F shown in Table 2.

Table 2. Signal positions (ppm) in ^1H - and ^{13}C -NMR spectra of the HMP and I-1 fractions

Substance	Residue	H-1	H-2	H-3	H-4	H-5	H-6
HMP fraction [-A-B-] _n	A	5.38	4.58	4.33	4.98	4.52	1.34
	B*	5.40	4.48	4.38	4.03	4.40	1.40
I-1 fraction [-C(D)-E(F)-] _n	C	5.28	4.63	4.27	4.16	4.52	1.30
	D	5.27	4.65	4.40	5.50	4.63	1.15
	E*	5.27	4.60	4.23	4.03	4.64	1.40
	F	5.41	4.73	5.52	4.16	4.54	1.40
		C-1	C-2	C-3	C-4	C-5	C-6
HMP fraction [-A-B-] _n	A	100.2	76.0	74.4	81.2	68.9	16.8
	B*	99.1	77.5	68.7	84.0	69.8	16.8
I-1 fraction [-C(D)-E(F)-] _n	C	100.0	74.5	73.4	69.9	67.8	16.8
	D	100.0	74.8	?	70.7	67.3	16.8
	E*	94.0	74.5	68.3	83.6	68.8	16.7
	F	94.9	74.2	70.7	79.6	68.8	16.7

* Formally identical B and E residues in F₄ and I-1 molecules are surrounded differently and therefore are different in positions of several signals in the NMR spectra.

a similar fraction. The production by enzymatic hydrolysis of a relatively low-molecular-weight acetylated polymer with disposition of sulfate groups different from that in the initial substance may be explained on the suggestion that irregular regions of native fucoidan molecules should contain a number of completely non-sulfated (and, possibly, also non-acetylated) fucose residues and that the fucoidanase cleaved only glycosidic bonds of these residues. The low yield of the I-1 fraction was caused by a small number of such residues in the initial fucoidan, and it being a polymer was due to assembling in blocks of regions with the structure different from the regular structure of the polysaccharide. Obviously, enzymatic hydrolysis of glycosidic bonds in fucoidan from *Fucus distichus* was mainly prevented by the high extent of sulfation of its molecules. We will continue studies on the effect of this enzyme preparation on natural fucoidans with different carbohydrate chain structure and different disposition of sulfate groups, as well as on products of their chemical desulfation.

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