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Distribution of O-Glycosylhydrolases in Marine Invertebrates. Enzymes of the Marine Mollusk *Littorina kurila* That Catalyze Fucoidan Transformation

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Abstract—The distribution of O-glycosylhydrolases (fucoidan hydrolases, α-D-mannosidases, β-D-glucosidases, and β-D-galactosidases) in 30 species of marine invertebrates occurring in the Sea of Japan was studied. It is shown that fucoidanases and glycosidases are widespread in the animals analyzed. Some molluscan, annelid, and echinoderm species can probably serve as objects for isolation and detailed study of the fucoidan-hydrolyzing enzymes. Fucoidan hydrolase, α-L-fucosidase, and arylsulfatase from the marine mollusk *Littorina kurila* were isolated and described. It was found that α-L-fucosidase and arylsulfatase hydrolyze synthetic substrates and cannot hydrolyze natural fucoidan, whereas fucoidan hydrolase cleaves fucoidan to produce sulfated oligosaccharides and fucose.

Key words: O-glycosylhydrolases, marine invertebrates, fucoidan, *Littorina kurila*, fucoidan hydrolase, α-L-fucosidase, arylsulfatase

Brown algae are a rich and renewable source of polysaccharides of various structure and biological activity. Besides the widely known alginic acid, they contain water-soluble polysaccharides laminarans (1,3; 1,6-β-D-glucanes) and fucoidans. Fucoidans represent a family of sulfated homo- and heteropolysaccharides isolated from many brown algal species. Besides fucose, these polysaccharides may contain glucose, galactose, mannose, xylose, rhamnose, and uronic acids. Although fucoidans exhibit a broad spectrum of biological activities—anticoagulative, antithrombotic, and antiviral (including antiHIV, antihepatitis, and antiherpes activities)—the details of their chemical structure remain to be determined [1-3].

The enzymes with known specificity that catalyze fucoidan hydrolysis (fucoidan hydrolases (or fucoidanases), sulfatases, and glycosidases) are important tools for studies of the structural characteristics of this group of polysaccharides and their biological role. Enzymatic transformation of polysaccharides by the enzymes from marine sources is of special interest. It allows obtaining new biologically active compounds differing from the original polysaccharides. For example, enzymatic transformation of laminaran yields a new glucan that exhibits antitumor and radioprotective properties [4]. It cannot be ruled out that the products of enzymatic degradation of fucoidans with simpler structure may retain biological activity of the complex molecule of the polysaccharide. It has been already shown that sulfated oligosaccharides—the products of fucoidan hydrolysis catalyzed by fucoidan hydrolase from the marine mollusk *Patinopecten yessoensis*—display anticoagulative activity similar to that of the original fucoidan [5].

It was shown earlier that fucoidan hydrolases, along with other carbohydrases, is contained in the hepatopancreas extract of the marine mollusk *Littorina* sp. [6]. A similar complex of enzymes was found in the crystalline style of the mollusk *Telescopium telescopium* [7]. To date, several fucoidan hydrolases have been isolated from marine microorganisms [8, 9] and invertebrates (the mollusks *Haliotus* sp. [10] and *P. yessoensis* [5] and the sea urchin *Strongylocentrotus nudus* [11]).

This work describes a search for sources of O-glycosylhydrolases in marine invertebrates and analysis of the complex of enzymes that catalyze fucoidan transformation in the gastropod *Littorina kurila*.

MATERIALS AND METHODS

Analytical methods. Neutral saccharides were determined by the phenol–sulfuric-acid method [12]; reduc-

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ing saccharides, by the method of Nelson [13]. The oligosaccharide composition was analyzed on a Jeol-JLC-6AH liquid chromatograph (Jeol, Japan) on a column packed with Biogel P-2 (1 × 100 cm), the oligosaccharides were detected with orcin–sulfuric-acid reagent. The saccharides were eluted from the column with 0.02 M acetate buffer (pH 5.4) at the rate of 16 ml/h.

The monosaccharide composition was determined by HPLC on a LC-5001 carbohydrate analyzer equipped with a Durrum DA-X8-11 column (385 × 3.2 mm) (Biotronik, Germany). Monosaccharides were detected using the bicinchoninate method. The C-R2 AX integration system was from Shimadzu, Japan.

The protein content in preparations was determined by the method of Lowry [14].

Substrates. Fucoidans from brown algae *Laminaria cichorioides* and *Fucus evanescens*, laminaran from *L. cichorioides*, and pustulan from the lichen *Umbellicaria russica* were obtained as described earlier [15, 16]. Carboxymethylcellulose, amylose, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl α-D-mannopyranoside, *p*-nitrophenyl α-L-fucopyranoside, *p*-nitrophenyl sulfate were from Sigma (USA).

Determination of enzymatic activity. The activity of the enzymes that catalyze hydrolysis of polysaccharides (amylopectin, CM-cellulose, laminaran, fucoidan, and pustulan) was estimated by an increase in the amount of reducing saccharides determined by the method of Nelson [13]. The incubation medium contained 100 μl of the enzyme, 200 μl of the corresponding substrate (4 mg/ml of fucoidan or 1 mg/ml of other polysaccharides), and 200 μl of 0.05 M succinate buffer, pH 5.4, supplemented with 0.2 M NaCl. The incubation duration did not exceed the time required for hydrolysis of 10% of the substrate contained in the incubation mixture.

The activity of glycosidases (with the *p*-nitrophenyl derivatives of the corresponding saccharides used as substrates) was estimated by the amount of *p*-nitrophenol produced [17].

The amount of the enzyme that catalyzed formation of 1 nmol of product (fucose, glucose, or *p*-nitrophenol) during 1 h was taken as an activity unit.

Isolation of enzymes. *Preparation of extracts of marine invertebrates*. Marine invertebrates were collected in Pos'eta Bay of Khasan Region of Primorskii Krai in August 2000 during an expedition of the research ship "Academician Oparin".

The specimens of each tissue (the hepatopancreas and the gut of the majority of animals, the crystalline styles of large mollusks, and smooth tissues of small species) taken from three animals were pooled, homogenized, and extracted with 0.05 M succinate buffer (pH 5.4) at a ratio of 1 : 3. The extract was centrifuged at 6000 rpm for 15 min, and the supernatant (1 ml) was gel filtered on a column with Sephadex G-25 (1 × 4 cm; Pharmacia, Sweden) to remove the low-molecular-weight contaminants. All procedures were performed at 4°C.

Isolation of fucoidan hydrolase, α-L-fucosidase, and arylsulfatase from the hepatopancreas of L. kurila. The molluscan hepatopancreas (100 g) was homogenized at 15,000 rpm for 30 sec and extracted with 0.1 M succinate buffer (pH 5.4). The extract was centrifuged at 12,000 rpm for 20 min, and the supernatant was mixed with ammonium sulfate to 90% saturation. The pellet was separated by centrifugation and dissolved in the minimum volume of 0.05 M succinate buffer (pH 5.4) containing 0.2 M NaCl (preparation A).

To isolate fucoidan hydrolase, preparation A was applied onto a column (2 × 10 cm) packed with a hydrophobic carrier phenyl-Sepharose (Pharmacia) equilibrated in 0.05 M succinate buffer (pH 5.4) containing 2 M $(NH_4)_2SO_4$. The protein was eluted using a stepwise gradient of $(NH_4)_2SO_4$ (2, 1, 0.5, and 0.05 M). The fractions with fucoidan hydrolase activity were pooled and desalted on a column with Sephadex G-25 (3 × 70 cm).

To isolate arylsulfatase, preparation A was dialyzed consecutively against distilled water and 0.05 M succinate buffer (pH 5.4), applied onto a column (1.5 × 15 cm) with SP-Sephadex (Pharmacia) equilibrated in the same buffer, and eluted with a NaCl linear gradient (from 0 to 1 M; 500 ml). The fractions containing arylsulfatase were pooled, dialyzed against the buffer, and chromatographed on a column packed with CM-cellulose (1.5 × 15 cm) (Whatman, UK). The proteins were eluted with a NaCl linear gradient (from 0 to 1 M; 500 ml). The arylsulfatase-containing fractions were pooled, concentrated by ultrafiltration on a PM-10 membrane (Amicon, Holland) to 30 ml, applied onto a column packed with Sepharose CL-6B (3 × 90 cm) (Pharmacia), and eluted with the buffer.

To isolate α-L-fucosidase, preparation A was chromatographed twice on SP-Sephadex under the conditions described above.

Determination of pH optimum of enzymes. The reaction mixture containing 100 μl of the enzyme, 200 μl of the corresponding substrate (4 mg/ml of fucoidan or 1 mg/ml of other polysaccharides), and 200 μl of succinate (pH 4.0-5.4), phosphate (pH 6.0-7.2), or borate (pH 7.5-9.0) buffer was incubated at 37°C. The enzymatic activity was assayed as described above.

Determination of temperature stability of fucoidan hydrolase. The enzyme (50 μl) in 0.05 M succinate buffer (pH 5.4) supplemented with 0.2 M NaCl was incubated for 30 min at temperatures from 0 to 70°C (with a 5-degree step). Thereafter, the substrate was added to the cooled samples, and the residual activity was determined.

Preparation of products of fucoidan hydrolysis. Dry fucoidan (200 mg) was added to the fucoidan hydrolase solution (20 ml) prepared in 0.05 M succinate buffer (pH 5.4) supplemented with 0.2 M NaCl or in 0.02 M

borate buffer (pH 8.5). The fucoidan hydrolase was prepared according to the scheme shown in Fig. 1. After the substrate was dissolved, the mixture was incubated at 37°C for 72 h. The reaction was stopped by boiling. The high-molecular-weight reaction products were separated by precipitation with ethanol added at a ratio of 1 : 4 (v/v). The fraction containing the low-molecular-weight reaction products was evaporated until dry under vacuum and analyzed on a Jeol-JLC-6 AH automatic liquid analyzer (Jeol).

RESULTS AND DISCUSSION

Systematic studies on the distribution of the carbohydrate-exchange enzymes in marine macro- and microorganisms have been performed for several years in the Laboratory of Enzyme Chemistry of the Pacific Institute of Bioorganic Chemistry [18]. Earlier, we published data on the content of fucosidases and some glycosidases in the digestive organs of 33 most widespread species of marine invertebrates occurring in Troitsa Bay of the Sea of Japan near the Marine Experimental Station of the Pacific Institute of Bioorganic Chemistry [19]. During the expedition on the research ship "Academician Oparin" in Pos'eta Bay, we considerably extended the list of animals studied. Thirty representatives of different classes of marine invertebrates (mollusks, starfishes, ophiurans, sea anemones, and worms) were caught mostly by dragging.

The results of analysis of the fucoidan hydrolase and glycosidase activities in the digestive organs of these animals (Table 1) confirmed the conclusions made in [19] and showed that fucoidan hydrolases and glycosidases are contained in practically all of the studied materials. Note that the fucoidan hydrolase activity in invertebrates is much lower that the activities of other O-glycosylhydrolases—1,3-β-D-glucanases [20], cellulases and chitinases [21, 22], and glycosidases (Table 1). This trend is also characteristic of fucoidan hydrolases from marine microorganisms [23]. The types Coelenterata (coelenterates) and Annelida (true worms), which displayed high fucoidan hydrolase activity (up to 235 units in *Chaetopterus cautus*), had not been analyzed in [19].

It is commonly believed that a low activity of the carbohydrate-hydrolyzing enzymes in the digestive organs of starfishes is related to their way of feeding (starfishes are predators and contain active proteinases) [24]. The glycosidase activity recorded in the starfishes *Leptosterias arctica* and *Distolasterias aligans* was comparable to that recorded earlier in *Asterias amurensis*, *Lysastrosoma anthosticta*, *Patiria pectinifera*, and *Gistolasterias nipon* [19]; conversely, the fucoidan hydrolase activity was fairly high (149 and 160 units, respectively) (Table 1).

Thus, this study showed that fucoidan hydrolases and glycosidases are widespread in marine invertebrates. The representatives of mollusks, worms, and echinoderms may be objects for isolation and thorough study of the fucoidan-hydrolyzing enzymes.

The gastropod *L. kurila*, which is abundant in the littoral area and is able to tolerate long-term transportation in sea water, was chosen as a source of fucoidan-degrading enzymes. The study of the composition and activity of various O-glycosylhydrolases from the hepatopancreas of *L. kurila* showed that it contains highly active glycosidases (β-D-galactosidase, α-D-mannosidase, β-D-glucosidase, sulfatases, and α-L-fucosidase) and glucanases (1,3-β-D-glucanase, amylase, and cellulase) (Table 2). The activity of fucoidan hydrolase was by an order of magnitude lower than the activities of other polysaccharide-hydrolyzing enzymes.

To select the conditions for isolation of enzymes that could be involved in the fucoidan degradation, we used a large set of cation- and anion-exchange carriers (SP-Sephadex, DEAE-Sephadex, CM-cellulose, DEAE-cellulose, DEAE-Toyopearl, and DEAE-Sepharose). Note that fucoidan hydrolase was not trapped on any sorbent used. Figure 1 shows the sequence of the procedures of separation and purification of fucoidan hydrolase, α-fucosidase, and arylsulfatase, which was developed in this study.

This scheme allowed us to separate fucoidan hydrolase from the other enzymes that may be involved in the fucoidan transformation. In most studies on the fucoidan-degrading enzymes, partially purified enzymes were used. Fucoidan hydrolase, α-fucosidase, and arylsulfatase from the hepatopancreas of *P. yessoensis* were separated by Japanese researchers [5].

When determining the pH optimum of fucoidan hydrolase, we obtained two distinct peaks with maxima at 5.4 and 8.5 (Fig. 2), which is suggestive of existence of two forms of this enzyme. Note that the optimum pH values for the majority of fucoidan-hydrolyzing enzymes studied are in acidic [11], slightly acidic, or neutral pH range [5, 9, 10]. Fucoidan hydrolases with the pH optima of 8 and more have not been found.

The fucoidan hydrolase isolated in this study completely lost its activity when stored in 0.05 M succinate buffer (pH 5.4) at 4°C for a week. The enzyme stability increased in the presence of NaCl: at the optimum NaCl concentration (0.2 M) the enzyme remained active for a month. The alkaline form of fucoidan hydrolase is less tolerant to NaCl. The dependence of activity of the two forms of fucoidan hydrolase on the NaCl concentration is shown in Fig. 3. The effect of temperature on the enzyme activity is shown in Fig. 4.

The fraction of low-molecular-weight final products of fucosidase action on fucoidan from *F. evanescens* at pH 5.4 was analyzed by chromatography on an automatic liquid analyzer. The fraction of low-molecular-weight products was obtained after precipitation of the high-molecular-weight fucoidan fragments with 80% aqueous

Table 1. Fucoidan hydrolase and glycosidase activities in marine invertebrates

Type, class, species (family)	Organ	Fucoidan hydrolase activity*	Glycosidase activity**		
		fucoidan from *Fucus evanescens*	*p*-Nph-Glc	*p*-Nph-Gal	*p*-Nph-Man
COELENTERATA					
Anthozoa					
Actinia sp.	DT	43	162	278	290
Cnidopulus japonicus	all	19	141	516	557
Anthopleura orientalis	all	27	103	239	116
Tialia fellina	all	38	122	313	1450
ANNELIDA					
Polichaeta					
Palynoidae	all	0	71	91	135
Sabelidae	all	80	46	290	321
Sabelidae	all	187	112	82	89
Tubulamus punctatus	all	28	71	159	106
Chaetopterus cautus	all	235	2044	3268	647
Eudistylial polymorpha (Bispira)	all	117	690	495	204
Sipunculida phascolosoma	all	80	316	888	395
NEMERTINI					
Nemertini					
Collarenemertes bimaculata	proboscis	105	0	524	0
ARTHROPODA					
Crustacea					
Pandalus hypsiuotus	HP	0	278	1564	222
Chiohoecetes opilio elongatus	HP	0	690	1525	313
Pagurus beringanus	all	32	0	0	428
MOLLUSCA					
Monoplacophora					
Onchidiopsis sp.	HP	86	296	919	140
Tritia tratercula	HP	0	982	1111	1174
Plicifucus plicatus	HP	7	766	201	126
Neptunea bulbacea	HP	2	0	1292	0
Neptunea lyrata	HP	0	0	0	0
Astarte borealis	HP	33	402	390	139
Lussiovolutopsius sp.	HP	18	383	623	322
Bivalvia					
Cyclocardia rjabininae	HP	41	108	285	26
Modiolus difficilus	CS	14	67	78	14
	HP	38	502	828	163
ECHINODERMATA					
Ophiuroidea					
Ophiura sarsi	DT	0	0	1418	0
Echinoidea					
Strongylocentrotus pallidus	DT	31	1865	758	256
Asteroidea					
Leptosterias arctica	DT	149	538	387	232
Distolasterias aligans	DT	159	694	386	270
Hencricia sp.	DT	17	537	494	304
Crinodea	DT	92	368	261	306

* Specific activity was expressed in nmol of fucose/h per mg protein. The protein content was determined by the method of Lowry [14].

** Specific activity was expressed in nmol of *p*-nitrophenol/h per mg protein. Designations: DT, digestive tract; HP, hepatopancreas; CS, crystalline style; *p*-Nph-Glc, *p*-nitrophenyl β-D-glucopyranoside; *p*-Nph-Gal, *p*-nitrophenyl β-D-galactopyranoside; *p*-Nph-Man, *p*-nitrophenyl α-D-mannopyranoside.

Table 2. O-Glycosylhydrolases from hepatopancreas of *L. kurila*

Enzyme	Substrate	Activity, nmol/h per mg protein
Fucoidan hydrolase	fucoidan from *F. evanescens*	71
1,3-β-D-glucanase	laminaran from *L. cichorioides*	35 714
Amylase	amylopectin	11 309
Cellulase	CM-cellulose	15 550
Agarase	agar (galactoglycan)	1237
Pustulanase	1,6-β-D-glucan	1524
β-D-Galactosidase	*p*-Nph-b-D-galactopyranoside	11 103
β-D-Glucosidase	*p*-Nph-b-D-glucopyranoside	11 401
α-D-Mannosidase	*p*-Nph-a-D-mannopyranoside	3447
Arylsulfatase	*p*-Nph-sulfate	12 000
α-L-Fucosidase	*p*-Nph-a-L-fucopyranoside	4370

alcohol. The results of chromatography show that the reaction products represent a mixture of oligosaccharides and monomer (Fig. 5a). HPLC analysis showed that the monosaccharide is fucose.

A different distribution pattern was characteristic of the products obtained under similar conditions as a result of fucoidan hydrolase treatment of fucoidan from *L. cichorioides* (with a shift towards formation of more low-molecular-weight products) (Fig. 5b). This is due to the fact that the two fucoidans differ in the degree of sulfation and in the molecular weight [15]. The maximum extent of hydrolysis for both fucoidans (assessed by the yield of reducing saccharides) was 5-6%.

A preliminary chromatographic analysis showed that the spectrum of the products formed as a result of fucoidan hydrolase action on fucoidan from *F. evanescens* at pH 8.5 and 5.4 is different. A comparison of the proportion of the low- and high-molecular-weight products obtained as a result of hydrolysis of fucoidan from *L. cichorioides* is threefold lower compared to fucoidan from *F. evanescens* (Table 3). This is indicative of a higher specificity of fucoidan hydrolase with pH optimum at 5.4

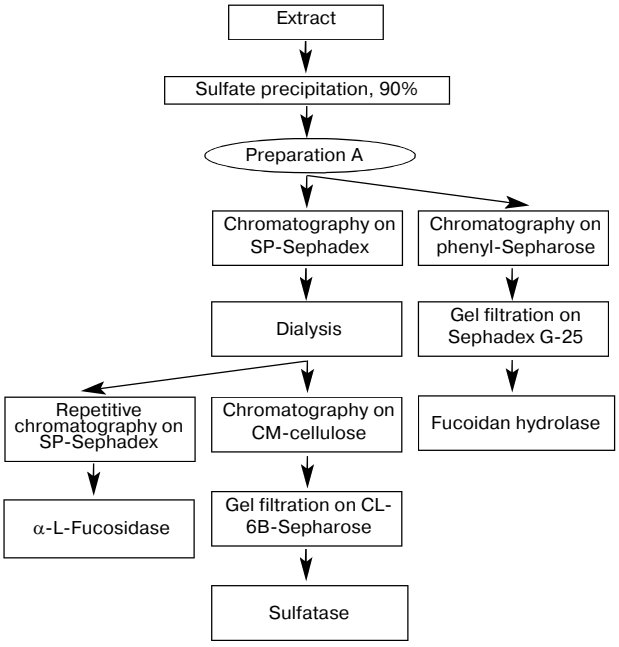

Fig. 1. Sequence of procedures of separation and purification of fucoidan hydrolase, α-fucosidase, and arylsulfatase from the hepatopancreas of the marine mollusk *L. kurila*.

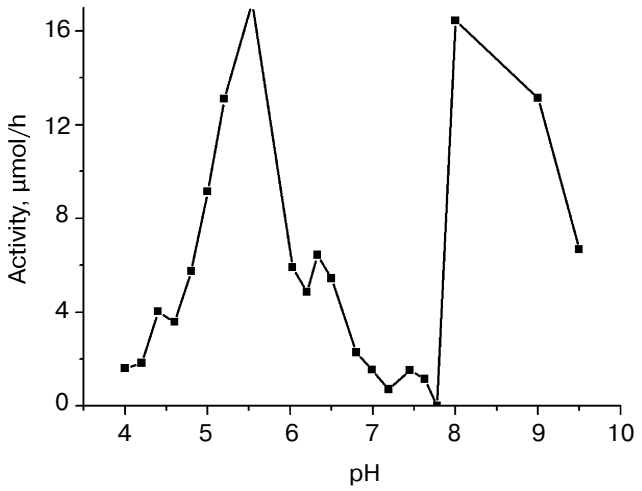

Fig. 2. pH dependence of the activity of fucosidase from *L. kurila*.

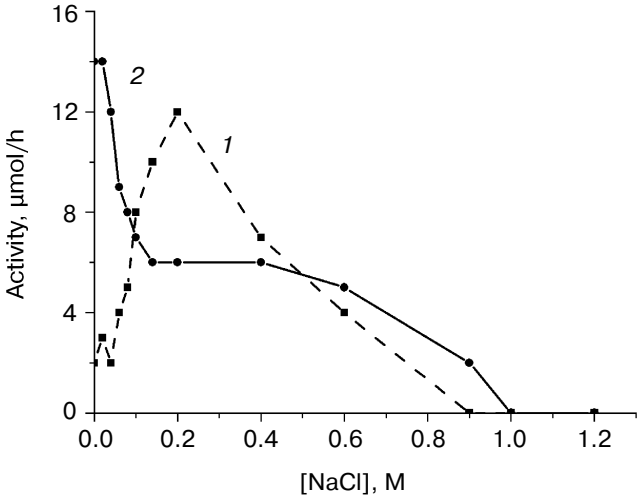

Fig. 3. Dependence of the activity of fucoidan hydrolase from *L. kurila* on NaCl concentration at pH 5.4 (*1*) and 8.4 (*2*).

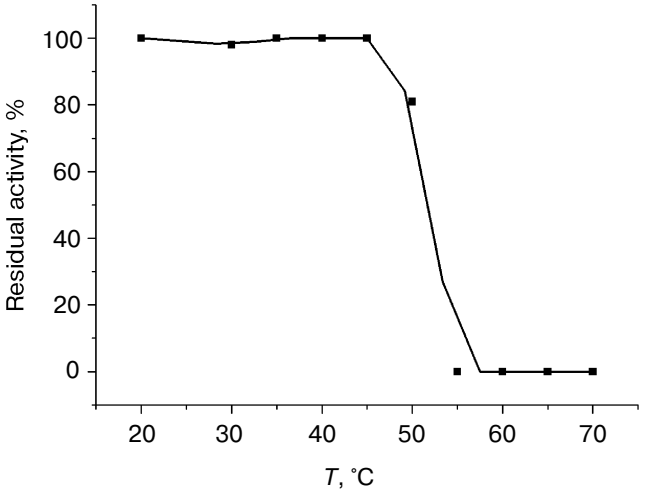

Fig. 4. Temperature stability of fucoidan hydrolase from *L. kurila* at pH 5.4 (0.05 M succinate buffer supplemented with 0.2 M NaCl).

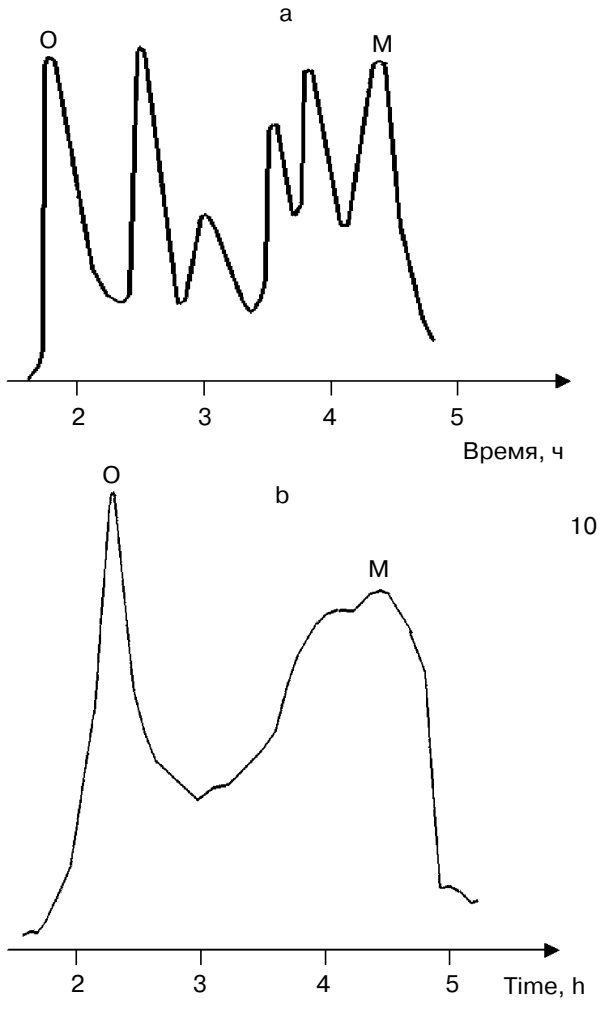

Fig. 5. Gel filtration of the products of hydrolysis of fucoidan from *F. evanescens* (a) and *L. cichorioides* (b) catalyzed by fucosidase at pH 5.4. Gel filtration was performed on a Jeol-JLC-6AH automatic liquid analyzer using Biogel P-2 as a carrier. Designations: M, monomer; O, oligosaccharides with $n \geq 7$.

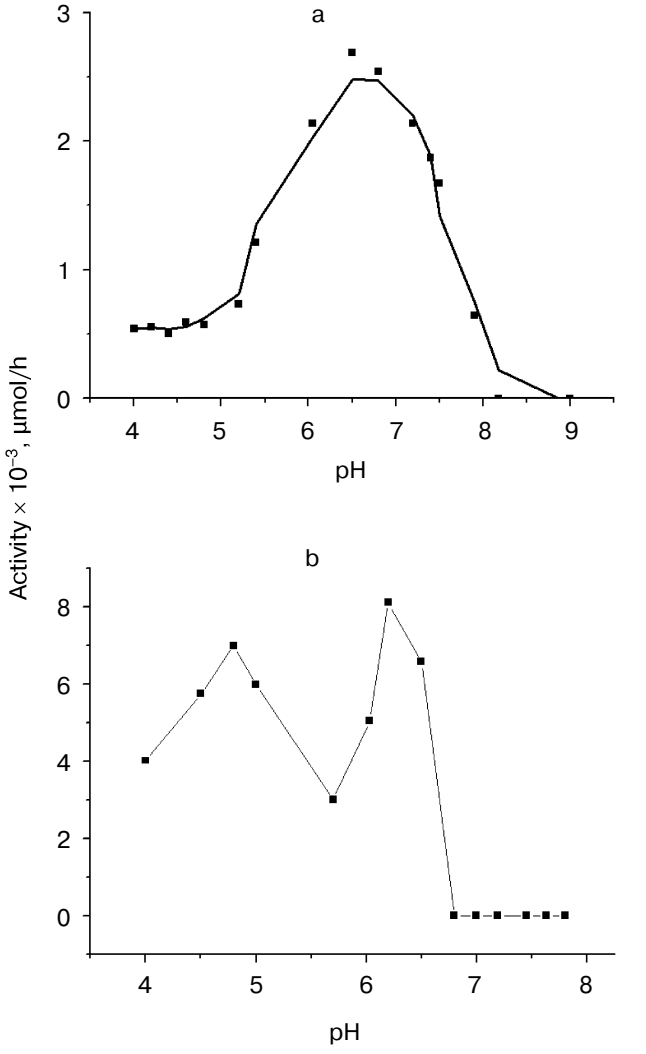

Fig. 6. Optimal pH for α-L-fucosidase (a) and arylsulfatase (b) from *L. kurila*.

Table 3. Characteristics of the products of enzymatic hydrolysis of fucoidans

Enzyme (pH)	Substrate	Yield of reaction products, %*	
		HMW products**	LMW products***
Fucoidan hydrolase (pH 5.4)	fucoidan from *F. evanescens*	85	15
Fucoidan hydrolase (pH 5.4)	fucoidan from *L. cichorioides*	95	5
Fucoidan hydrolase (pH 8.5)	fucoidan from *F. evanescens*	55	45

* Yield of the reaction products was evaluated using the phenol–sulfuric-acid method [12].
** High-molecular-weight products ($n \geq 7$).
*** Low-molecular-weight products (oligosaccharides, $n = 2$-7, fucose).

towards the low-sulfated fucoidan from *F. evanescens*. The yield of low-molecular-weight products formed as a result of hydrolysis of fucoidan from *F. evanescens* with alkaline fucoidan hydrolase was threefold greater than in the case of acid fucoidan hydrolase. This difference may be explained by the fact that the substrate may exist in the protonated and unprotonated forms.

Earlier, the studies of fucoidan enzymatic degradation revealed the existence of two types of α-L-fucosidases. The hepatopancreas of the marine mollusk *P. yessoensis* [5] contained both α-L-fucosidase that hydrolyzed only *p*-Np-α-L-fucoside but not fucoidan (type 1), and the enzyme that, besides the artificial substrate, also slowly hydrolyzed fucoidan to form fucose (type 2) (similar to α-L-fucosidase from *Fusarium oxysporum* [8]). Our studies showed that α-L-fucosidase from *L. kurila* with pH optimum 5.4 had no effect on natural fucoidan and, therefore, is a type 1 enzyme (Fig. 6a).

The arylsulfatase preparation isolated in this study from the hepatopancreas of *L. kurila* effectively hydrolyzed *p*-nitrophenyl sulfate but had no effect on fucoidan. The elution profiles of fucoidan from *F. evanescens* and arylsulfatase-treated fucoidan obtained on a column with DEAE-Toyopearl were identical.

The plot of dependence of the activity of arylsulfatase (obtained after chromatography on CM-cellulose, Fig. 1) on pH had two maxima (Fig. 6b), which suggests the existence of two forms of arylsulfatase. These two forms were separated by gel filtration on CL-6B-Sepharose.

It was shown earlier that the hepatopancreas of the marine mollusk *Heliotus* sp. contains arylsulfatase that hydrolyzes *p*-nitrophenyl sulfate [10]. In addition, partially purified preparation of fucoidan hydrolase from *Heliotus* sp. exhibited a weak sulfatase activity towards fucoidan and sulfated oligosaccharides but had no effect on *p*-nitrophenyl sulfate. The authors of [10] assumed that desulfation does not precede the fucoidan hydrolase action. They believe that sulfatase influences the already low-molecular-weight products (sulfated fucose and oligosaccharides). Our data show that the complexes of fucoidan-degrading enzymes in marine mollusks *L. kurila* and *Heliotus* sp. are similar in composition and properties.

Studies of the properties and specificity of the enzymes of this complex from *L. kurila* will be continued.

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