ISSN 0026-2617, Microbiology, 2012, Vol. 81, No. 3, pp. 367–372. © Pleiades Publishing, Ltd., 2012.
Original Russian Text © S.P. Ermakova, E.P. Ivanova, I.Yu. Bakunina, V.V. Mikhailov, T.N. Zvyagintseva, 2012, published in Mikrobiologiya, 2012, Vol. 81, No. 3, pp. 396–402.

EXPERIMENTAL ARTICLES

Effect of Brown Algae Metabolites on the Synthesis of O-Glycosyl Hydrolases by Bacteria Degrading the Thallus of *Fucus evanescens*

S. P. Ermakova[a,1], E. P. Ivanova[b], I. Yu. Bakunina[a], V. V. Mikhailov[a], and T. N. Zvyagintseva[a]

[a] *Elyakov Pacific Institute of Bioorganic Chemistry, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia*

[b] *Swinburne University of Technology, Melbourne, Australia*

Received May 30, 2011

Abstract—The effect of fucoidan, extractive substances from *Fucus evanescens* and the *Laminaria cichorioides* protein (an inhibitor of endo-1→3-β-D-glucanase) on the degradation of *F. evanescens* thallus and on the growth of bacteria involved in this process was studied. The complex of O-glycosyl hydrolases and the level of their enzymatic activity in bacteria cultivated under various conditions depended significantly on the composition of the growth medium. The highest taxonomic diversity was observed for bacteria isolated from the thallus degraded in the control medium (sea water). These bacteria were characterized by very low levels of activity of the enzymes degrading polysaccharides (fucoidan, laminaran, and pustulan). In the presence of 1→3-β-D-glucanase inhibitors, the taxonomic diversity of the microorganisms degrading *F. evanescens* thallus was decreased, and the activity of O-glycosyl hydrolases (particularly, of fucoidan hydrolases) was increased.

Keywords: O-glycosyl hydrolases, brown algae, marine microorganisms, endo-1→3-β-D-glucanase inhibitor.

DOI: 10.1134/S0026261712030058

The vital activity of any organism requires efficient functioning of its enzymes. Understanding of the key role of enzymes in the cell processes resulted in their wide application in medicine, pharmacology, and toxicology. The action of many toxic substances is based on their ability to inhibit the enzymes; the same effect is typical for a number of medical preparations.

Inhibitors of O-glycosyl hydrolases isolated from natural sources are actively investigated. Natural inhibitors of α-amylase and glycosidase have been the best studied so far [1–4]. Participation of amylase inhibitors isolated from the cereals in plant/insect, plant/animal, and plant/microorganisms interactions has been investigated [5, 6].

Brown algae are a rich source of various polysaccharides: fucoidans, laminarans, and alginic acids [7–9]. Marine microorganisms of the algal phylloplane (epibionts) are able to degrade the thallus due to the complex of O-glycosyl hydrolases which depolymerize polysaccharides of the brown algae.

We previously demonstrated that brown algae contain the substances which were inhibited endo-1→3-β-D-glucanases of marine mollusks and microorganisms [10]. These enzymes are digestive: they catalyze the hydrolysis of laminarans—1→3;1→6-β-D-glucans, reserve polysaccharides of the brown algae [7, 11, 12]. From the brown algae *Laminaria cichorioides*, we isolated the protein inhibitor of irreversible type of action, which was highly specific for the endo-1→3-β-D-glucanases of marine mollusks: its inhibiting activity ($I_{50} \sim 10^{-8}$ M) was comparable with that of plant inhibitors of α-amylase from the digestive tract of herbivores [10]. It is important to reveal the role of such substances in algae/marine microorganisms' relations.

The enzymatic complex includes, among others, 1→3-β-D-glucanases, alginate lyases, α-L-fucosidases, α- and β-D-galactosidases, and β-D-glucosidases [13]. The activity of fucoidan hydrolases (the enzymes' degrading fucoidans, other widespread polysaccharides from the cell wall of brown algae, belonging to the family of sulfated α-L-fucans and heteropolysaccharides and possessing diverse biological activity [14, 15]) in marine organisms is extremely low [16]. The data regarding the isolation and investigation of fucoidanases, including fucoidanase from *Flavobacterium* sp. SA-0082 [17], are very scarce. Fucoidanases isolated from these bacteria are already used in the production of medical prophylactic beverages for depolymerization of fucoidan in order to increase their biological activity [17]. The data regarding natural inhibitors of these enzymes are not available.

The goal of this work was to study microbial degradation of *Fucus evanescens*, the microbial community

[1] Corresponding author; e-mail: swetlana_e@mail.ru

and enzyme activity of symbiotrophic bacteria participating in the degradation of the algal thallus in seawater, and to investigate the effect of fucoidan, extractive substances from *F. evanescens*, and the proteinaceous inhibitor of endo-1→3-β-D-glucanase from *Laminaria cichorioides* on the degradation of *F. evanescens* thallus.

MATERIALS AND METHODS

Analytical methods. Neutral sugars from bacterial biomass were determined by the phenol–sulfuric acid method [18], and the reducing ability was determined as described by Nelson [19].

Substrates. The enzymatic activity of bacteria was determined using the following substrates. Laminaran and fucoidan from brown algae *L. cichorioides* and pustulan from the lichen *Umbilicaria rossica* were obtained as described [15]. The commercial preparations were: agar and *p*-nitrophenyl derivatives of β-D-gluco-, β-D-N-acetylgluco-, a-D-manno-, a- and β-D-galacto-, a-L-fuco-, β-D-xylopyranosides (*p*NO_2Ph-β-D-Glc, *p*NO_2Ph-β-D-NAcGlc, *p*NO_2Ph-a-D-Man, *p*NO_2Ph-a- and β-D-Gal, *p*NO_2Ph-a-L-Fuc, *p*NO_2Ph-β-D-Xyl, respectively).

Algal extracts and metabolites. The algae were collected during the expeditions of research vessel *Academician Oparin* (*F. evanescens*, Iturup Island, the Sea of Okhotsk) and at the Marine Experimental Station of the Elyakov Pacific Institute of Bioorganic Chemistry, FEB RAS (*L. cichorioides*, Hasan region, Primorski krai) in August and September of 1999. The fresh or frozen algae were homogenized and treated with ethanol (1 : 1) at room temperature for two weeks. Ethanol extracts were vacuum-dried, and the dry pellet was used for the following study. Fucoidan from *F. evanescens* was isolated as described [20]. The proteinaceous inhibitor was isolated from the water–ethanol extract of *L. cichorioides* as described earlier [10].

Cultivation of microorganisms. Isolation of microorganisms from the degraded samples of *F. evanescens* thallus and determination of their taxonomic position were carried out by the method [21] with modifications. For degradation of the thallus, the pieces of *F. evanescens* (5 g) were washed with sterile seawater and placed into the glass flasks containing 200 mL of sterile culture medium. The control medium (medium K) was seawater at pH 7.5–7.8. Experimental media contained medium K and the following: fucoidan, 1 g/L (medium 1), proteinaceous inhibitor of laminarinases from *L. cichorioides*, 0.4 mg/L (medium 2), extractive substances from *F. evanescens*, 0.4 mg/L (medium 3). The flasks were incubated under static conditions at 25°C for two months.

Isolation of bacterial monocultures was carried out on solid media by using a stereoscopic microscope (Carl Zeiss) with 1000× magnification. The cultivation was carried out at 20–22°C. Bacteria were identified according to the standard methods and as described earlier [22, 23]. Pure cultures were maintained in semiliquid agar medium (5 g/L of agar) under mineral oil at 4°C and in liquid culture medium with 20% of glycerol, at –80°C. The strains KMM 3553 and KMM 3773 were deposited to the Collection of Marine Microorganisms, Elyakov Pacific Institute of Bioorganic Chemistry, Far Eastern Branch, the Russian Academy of Sciences, Vladivostok.

For primary screening of the enzymes, bacteria were grown in the medium containing the following (g/L): bactopeptone (Difco), 5.0; yeast extract (Difco), 2.0; K_2HPO_4, 0.2; $MgSO_4$, 0.05; seawater, 100 mL; pH 7.5–7.8 (control, medium K). Experimental media contained control medium with the following: fucoidan, 1 g/L (medium 1); proteinaceous inhibitor of laminarases from *L. cichorioides*, 0.4 mg/L (medium 2); extractive substances from *F. evanescens*, 0.4 mg/L (medium 3). Bacteria were grown on a rotary shaker (160 rpm) at 28°C.

Determination of the enzyme activity. The bacterial extract (0.01 mL) was supplemented with the solution of the relevant substrate (0.1 mL, 1 mg/mL) and 0.14 mL of 0.05 M sodium phosphate buffer (pH 7.0). The mixture was incubated at 37°C for 1–4 h. Activity of the enzymes hydrolyzing polysaccharides (fucoidan, laminaran, and pustulan) was defined as an increase in the amount of reducing sugars determined by the Nelson' method. Activity of glycosydases (with *p*-nitrophenyl derivatives of the corresponding sugars as the substrates) was determined by production of *p*-nitrophenol [24]. The activity unit was defined as the amount of the enzyme able to catalyze glucose formation (1 μmol) or *p*-nitrophenol (1 μmol) per 1 min under experimental conditions.

Inhibition of bacterial laminarinases. 0.01 mL of the solution of the proteinaceous inhibitor from *L. cichorioides* (1 μg) in 0.05 M phosphate buffer (pH 7.0) with addition of 0.01 μL of the enzyme (10^{-2} U/mL) was incubated at 37°C for 10 min, then 0.1 mL of laminaran (1 mg/mL) and 0.13 mL of 0.05 M phosphate buffer (pH 7.0) were added. The mixture was incubated at 37°C for 30 min, and the residual enzyme activity was determined by measuring the amount of the reducing sugars. Determination of the inhibitors was carried out using (1→3-β-D-glucanase L_{IV} from *Spisula sachalinensis* (10^{-2} U/mL) as the positive control [10].

Bacterial extracts. Bacterial biomass (0.4 g) was resuspended in 3 mL of 0.05 M sodium phosphate buffer (pH 7.0) and sonicated in a UZND-2 sonicator. The obtained suspension was centrifuged at 10000*g* for 30 min at 4°C. The content of laminarinase, agarase, fucoidan hydrolase, pustulanase, α-galactosidase, β-galactosidase, β-glycosidase, β-N-acetylglucosaminidase, α-mannosidase, α-fucosidase, β-xylosidase activities in the supernatant was determined as described above.

Isolation of laminarinase. Bacterial extracts (5 mL) were fractionated on a G-75 Sephadex column (50 × 1 cm) with 0.05 M phosphate buffer (pH 7.0) and eluted with the same buffer (0.3 mL/min). The enzymatic activity of the fractions was determined. The active fractions were combined and used in the study of laminarinase inhibition (as described earlier).

RESULTS AND DISCUSSION

The effect of various metabolites of the brown alga *F. evanescens* on the growth of bacteria degrading its thallus was investigated. Microbial degradation of *Fucus evanescens* in seawater (medium K) was analyzed. The effect of fucoidan (medium 1), proteinaceous inhibitor of laminarases from *L. cichorioides* (medium 2), and extractive substances from *F. evanescens* (medium 3) added to the medium K on the composition and properties of O-glycosyl hydrolases from marine microorganisms participating in the thallus degradation was studied. The samples of *F. evanescens* thallus in control and experimental media were incubated at room temperature for 2 months until their complete degradation.

Taxonomic studies of marine bacteria isolated from the samples of four experimental groups after 2 months of incubation demonstrated that four phenotypically diverse bacteria species prevailed in the control group (Table 1; working numbers of the strains are shown without designation of the species). Notably, the activity of polysaccharide-degrading enzymes in the thallus was very low or completely absent. Three phenotypically diverse groups of bacteria were isolated from the samples with fucoidan and the *L. cichorioides* inhibitor. In the presence of extractive substances from *F. evanescens*, only two groups of bacteria were registered (Table 1). Thus, the presence of 1→3-β-D-glucanase inhibitors led to a decrease in the number of dominating groups of bacteria.

From each dominating phenotype, bacterial strains were isolated and the content of some O-glycosyl hydrolases was investigated. The composition of O-glycosyl hydrolases and their activity in bacteria cultivated under different conditions depended significantly on the growth medium composition. The number of heterotrophic bacteria per mL of culture medium was 6.0×10^3 (medium K), 4.0×10^3 (medium 1), 5.5×10^3 (medium 2), and 7.0×10^3 (medium 3). In the control medium containing only the thallus of *F. evanescens*, predominance of bacteria that exhibited very low activity of the polysaccharide-degrading enzymes (including laminarinases) was observed (Table 2). In this "control" group, the strain identified as *Bacillus* sp. F12 had the highest level of 1→3-β-D-glucanase activity ($1.6 \text{ U} \times 10^{-2}$/g biomass) and significant level of β-D-glucosidase activity ($9 \text{ U} \times 10^{-2}$/g biomass) (Table 1). The addition of fucoidan did not affect the level of activity of microbial O-glycosyl hydrolases (Table 1).

When the metabolites of *L. cichorioides* (medium 2) and *F. evanescens* (medium 3) that inhibit 1→3-β-D-glucanases of mollusks and bacteria were present in the culture medium, synthesis of 1→3-β-D-glucanases, fucoidan hydrolases, glycosidases, and oligosaccharide-hydrolases (mainly, β-D-glucosidases, β-NAc-glucosaminidases, and β-D-xylosidases, more seldom—β-D-galactosidases with high level of activity) was induced in the bacterial isolates obtained in the present work (Table 1).

During degradation of the brown algae thallus in the presence of laminarinase inhibitors, the microorganisms demonstrating high activity of the polysaccharide-degrading enzymes were probably predominantly developed (Table 1). *Formosa* sp. strains F44, F45, F80, F89 were shown to possess high levels of fucoidan hydrolase activity (Table 1). In these strains, fucoidan hydrolase activity was comparable to 1→3-β-D-glucanase activity. Such high levels of fucoidan hydrolase activity have not been previously reported.

Using molecular biological methods, strain F89, which synthesized the most active fucoidanase, was identified as *Formosa algae*, family *Flavobacteriaceae* (KMM 3553) [25].

To determine whether the presence of algal metabolites inhibiting 1→3-β-D-glucanases promoted selective development of the microorganisms with high activity of the polysaccharide-degrading enzymes, we undertook the comparative study of the properties of laminarinases from strains KMM 3773 (medium K) and KMM 3553 (medium 2). These strains were shown to possess the highest 1→3-β-D-glucanase activity in their control groups (Table 1). For comparative study, the strains KMM 3773 and KMM 3553 were grown on the media K and 2. Biomass of the strains grown in the presence of *L. cichorioides* proteinaceous inhibitor (medium 2) was approximately 1.5–2 times higher than that of the strains grown in the medium K (Table 2). Thus, the growth of both strains was not repressed by this proteinaceous inhibitor, so direct toxic effect of the algal metabolites on these microorganisms was excluded. The enzymatic activity of strain KMM 3553 was higher than that of KMM 3773 in culture media. For these strains, induction of 1→3-β-D-glucanase in the presence of the *L. cichorioides* protein was shown. As previously, fucoidan hydrolase was found only in the biomass of strain KMM 3553; similar to 1→3-β-D-glucanase, it was induced in the presence of the *L. cichorioides* inhibitor (Table 2).

The proteinaceous inhibitor from *L. cichorioides* in vitro was found to dramatically inhibit the activity of partially purified preparations of 1→3-β-D-glucanase from KMM 3773. At the same time, this protein did not affect 1→3-β-D-glucanase activity from KMM 3553 (Table 2).

Table 1. Activity of O-glycosyl hydrolases of bacteria associated with the brown alga *Fucus evanescens* and cultivated in various culture media

Growth medium	Phenotype	Working no. of the strain	O-glycosyl hydrolase activity, U × 10^{-2}/g biomass										
			1	2	3	4	5	6	7	8	9	10	11
Control medium (medium K)	I	F7	–*	–	–	–	–	–	–	–	–	–	9
		F 8	–	–	–	–	–	–	–	–	–	–	11
		F 11	–	–	–	–	–	–	–	–	–	–	43
		F 56	–	–	–	–	–	–	–	–	–	–	10
		F 57	–	–	–	–	–	–	–	–	–	–	–
		F 58	–	–	–	0.4	–	–	–	10	–	–	–
	II	F 12	1.6	–	–	–	–	–	9	–	–	–	–
	III	F 13	–	–	–	0.9	–	–	11	11	–	–	–
		F 59	–	–	–	–	9	42	11	8	–	–	42
	IV	F 1	1.2	–	–	–	41	43	41	10	–	–	–
		F 3	0.8	–	–	–	–	–	–	40	–	–	–
		F 10	1.4	–	–	0.3	–	–	–	–	–	–	–
Medium with fucoidan (medium 1)	V	F 14	–	–	–	–	–	–	10	9	–	–	–
		F 15	–	–	–	–	–	–	–	–	–	–	–
		F 61	0.5	–	–	–	–	–	23	8	13	–	14
		F 62	2.0	–	–	–	–	–	9	10	–	10	48
		F 67	–	–	–	–	–	–	11	9	–	9	10
	II	F 23	0.4	–	–	–	–	–	–	10	–	–	–
		F 24	–	–	–	–	–	–	–	7	–	–	–
		F 25	0.4	–	1.0	–	–	–	–	12	–	–	–
		F 70	0.5	–	0.3	–	–	–	–	–	–	–	–
		F 72	–	–	–	–	–	–	–	–	–	–	–
		F 73	–	–	–	–	–	–	–	–	–	–	–
	IV	F 65	1.0	–	–	–	10	41	25	22	10	9	43
Medium with the proteinaceous inhibitor from *Laminaria cichorioides* (medium 2)	V	F 43	1.9	0.7	–	–	–	–	44	10	–	9	43
		F 44	2.6	1.9	1.5	–	–	–	40	9	12	11	10
		F 45	1.7	1.9	2.0	–	–	–	12	14	11	16	9
		F 89	2.9	0.5	2.8	–	–	–	14	13	9	12	45
		F 90	1.0	0.6	–	–	–	–	220	270	15	8	10
	I	F 36g	0.3	0.5	–	–	–	–	45	11	10	10	9
		F 36p	–	–	–	–	–	42	9	43	–	–	220
		F 37g	–	–	–	–	–	45	7	41	8	–	240
	II	F 88	0.4		0.6	–	–	–	–	–	–	–	–
		F 93	1.9	1.2	1.2	–	–	–	280	44	9	–	10
		F 94	0.3	–	–	–	–	–	210	40	10	12	9
Medium with extractive substances from *Fucus evanescens* (medium 3)	V	F 77	–	–	0.3	–	–	–	9	10	–	–	9
		F 78	2.3	–	0.6	–	–	10	11	9	–	12	13
		F 79	0.5	–	1.6	–	10	43	42	14	11	–	10
		F 80	5.9	–	1.3	–	–	–	9	10	–	–	47
		F 81	1.8	0.8	1.0	–	–	–	40	8	10	9	40
	II	F 82	0.4	–	1.6	–	–	–	11	9	–	–	–
		F 86	0.4	–	0.4	–	–	10	15	11	–	–	–
		F 28	–	–	–	0.5	–	–	8	10	–	–	–
		F 29	0.3	–	–	–	–	–	12	8	–	–	–
		F 74	0.8	0.5	0.5	–	–	–	10	12	–	–	–
		F 75	–	–		–	–	–	9	10	–	–	–
		F 76	–	–	1.1	–	10	10	43	9	–	–	42

Notes: The substrates used in the work: laminaran (1), agar (2), fucoidan (3), pustulan (4), and *p*-nitrophenil derivatives of α-D-Gal (5), β-D-Gal (6), β-D-Glc (7), β-D-NAcGlc (8), α-D-Man (9), α-L-Fuc (10), β-D-Xyl; (11).

"–" indicates no activity detected.

Table 2. Some characteristics of marine strains *Bacillus* sp. F12 and *Formosa algae* F89 cultivated in the media K and 2 and of the enzymes produced by these strains

Strain	Culture medium	Biomass, g/L	1→3-β-D-glucanase, U × 10^{-2}/g biomass	Fucoidan hydrolase, U × 10^{-2}/g biomass	Inhibition of 1→3-β-D-glucanase in vitro*
Bacillus sp. F12 (KMM 3773)	K	1.5	1.6	–**	93
	2	2.5	5.2	–**	95
	2/K***	1.6	3.3	–**	–
Formosa algae F89 (KMM 3553)	K	8.1	2.9	2.8	–
	2	17.9	5.6	7.8	–
	2/K***	2.2	1.9	2.7	–

Notes: * Per cent from the initial enzyme activity.
** Absence of the enzymatic activity or inhibition.
*** The ratio of the biomass and the enzyme activity in the medium K and medium 2.

Thus, the presence of the 1→3-β-D-glucanase inhibitor from *L. cichorioides* in the medium for the bacteria participating in the degradation of *F. evanescens* thallus induced synthesis of a number of O-glycosyl hydrolases and essentially enhanced their activity (Table 1). Activity of 1→3-β-D-glucanase of the strains *Bacillus* sp. F12 and *F. algae* F89 was increased in vivo in the presence of this inhibitor 3- and 2-fold, respectively, although the enzyme from *F. algae* was not sensitive to the inhibitor in vitro (Table 2). The substances from the brown algae induced 1→3-β-D-glucanase synthesis as well. Notably, they also induced synthesis of fucoidan hydrolase and β-D-gluco- and xylosidase, the enzymes involved in the degradation of the brown algae polysaccharides (Table 1). Selective development of these microorganisms in the presence of regulatory algal factors probably resulted from the high activity of their enzymes, insensitive to the natural inhibitors (Table 2). Moreover, the enzymes hydrolyzing algal polysaccharides were probably induced by the algal metabolites (including those with the inhibitory activity).

The present work is the part of a directed study of degradation of *F. evanescens* thallus [21]. An applied aspect of the study is the development of selective culture medium for the isolation of bacterial producers of fucoidan hydrolase. The literature data regarding the sources of fucoidan hydrolases are very scarce [26–28]. The usage of algal metabolites with inhibitory activity may make it possible to affect the synthesis of these enzymes.

One of the properties of extractive substances from the brown algae is inhibition of 1→3-β-D-glucanases. This study allowed us to also demonstrate the ability of these substances to stimulate the development of microorganisms degrading *F. evanescens* and thus to affect the taxonomic diversity of bacterial degraders. The microorganisms dominating in the media containing these substances had broader ranges of highly active O-glycosyl hydrolases participating in the degradation of the polysaccharides of brown algae. The studied inhibitors are probably the regulators of the algae–marine microorganisms interrelations, and therefore could be very interesting for microbial ecology.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research, project no. 12-04-00669, by the Far Eastern Branch of the Russian Academy of Sciences, project no. 12-III-A05-057, by the Molecular and Cell Biology Program of the Presidium of the Russian Academy of Sciences, and by the grant of the Ministry of Education and Science, project no. 02.740.11.0777.

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