Chemical Biology

Expression and biochemical characterization and substrate specificity of the fucoidanase from *Formosa algae*

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Abstract

A gene that encodes fucoidanase *ffa2* in the marine bacterium *Formosa algae* strain KMM 3553 was cloned, and the protein (FFA2) was produced in *Escherichia coli*. Recombinant fucoidanase FFA2 was purified, and the biochemical properties of this enzyme were studied. The amino acid sequence of FFA2 showed 57% identity with known fucoidanase FcnA from *Mariniflexile fucanivorans*. The mass of the gene product FFA2 is 101.2 kDa (918 amino acid residues). Sequence analysis has revealed that fucoidanase FFA2 belongs to the GH107 (CAZy) family. Detailed substrate specificity was studied by using fucoidans from brown seaweeds as well as synthetic fucooligosaccharide with distinct structures. Fucoidanase FFA2 catalyzes the cleavage of (1→4)-α-glycosidic bonds in the fucoidan from *Fucus evanescens* within a structural fragment (→3)-α-L-Fucp2S-(1→4)-α-L-Fucp2S-(1→)ₙ, but not in a fragment (→3)-α-L-Fucp2S,4S-(1→4)-α-L-Fucp2S-(1→)ₙ. Using synthetic di-, tetra- and octasaccharides built up of the alternative (1→4)- and (1→3)-linked α-L-Fucp2S units, the difference in substrate specificity and in the rate of enzymatic selectivity was investigated. Nonsulfated and persulfated synthetic oligosaccharides were not transformed by the enzyme. Therefore, FFA2 was specified as poly[(1→4)-α-L-fucoside-2-sulfate] glycanohydrolase. This enzyme could be used for the modification of natural fucoidans to obtain more regular and easier characterized derivatives useful for research and practical applications.

Key words: brown algae, fucoidan, substrate specificity, sulfated oligosaccharides, sulfated polysaccharides

Introduction

Anionic polysaccharide fucoidans from brown seaweeds and echinoderms are studied intensively due to their ability to influence various biological processes, such as inflammation, blood coagulation, angiogenesis and cell adhesion (Pomin 2012; Kwak 2014; Fitton et al. 2015). Fucoidans are built up mainly of sulfated α-L-fucose
residues linked through (1→2), (1→3)- and (1→4)-glycoside bonds. Types of bonds, pattern and degree of sulfation, as well as the presence of nonfucose units in a structure of fucoidans depend on algae/echinoderm species, region and time of harvesting, methods of isolation and purification (Cumashi et al. 2007; Ustyuzhanina et al. 2014a, 2014b). Because of heterogeneity and irregularity of fucoidans, it seems to be perspective to transform these biopolymers to more regular and well-characterized derivatives useful for research and practical applications. Therefore, chemical and/or enzymatic modifications of fucoidans are regarded as convenient approaches to the preparation of such compounds.

Fucoidanases are enzymes that catalyze the cleavage of glycoside bonds between fucose residues in fucoidan chains. These proteins belong to the class of EC 3.2.1. glycosidases. According to the Carbohydrate Active Enzyme database (http://www.cazy.org/) (Lombard et al. 2014), fucoidanases are included in the 107 family of glycosidases. These enzymes were found in marine organisms such as bacteria (Bakunina et al. 2000; Descamps et al. 2006; Silchenko et al. 2013), invertebrates (Kitamura et al. 1992; Daniel et al. 1999; Kusaykin et al. 2003; Bilan et al. 2005; Silchenko et al. 2014) and some fungi (Rodriguez-Jasso et al. 2010). Several attempts to fucoidan transformation by the extracts from different sources expressing fucoidanase activity have been performed to obtain low-molecular-weight derivatives (Fukuta 2008; Kim et al. 2008, 2010). Nevertheless, only a few studies regarding the isolation, characterization and determination of a substrate specificity of fucoidanases have been performed because of the lack of reference fucoidans or oligomeric substrates with distinct structure (Kusaykin et al. 2016). The nucleotide sequences of fucoidanases and their deduced amino acid sequences have been published only for *Marinoflexile fucanivorans* SW5T (Colin et al. 2006), *Alteromonas* sp. SN-1009 (Takayama et al. 2002) and *Shewanella violacea* DSS12T (GenBank: BAJ00350.1).

Previously, we reported on the isolation and initial characterization of catalytic properties of intracellular fucoidanase FFA from the marine bacteria *Fucus algeae* KMM 3553T (Silchenko et al. 2013). A partially purified enzyme was used to degrade fucoidan from brown algae *Fucus evanescens*. Based on the NMR analysis of the structure of the products, fucoidan FFA specifically cleaves (1→4)-bonds in fucoidan molecule built up of alternating (1→3)- and (1→4)-linked α-L-fucopyranose residues sulfated mainly at O-2. Unfortunately, due to complexity and structural irregularity of the fucoidan molecules, it was difficult to determine a precise substrate specificity of the fucoidanase, including influences of sulfation pattern and degree of polymerization. In this context, the model synthetic oligosaccharides that represent distinct structural fragments of fucoidans can be regarded as indispensable tools for the assessment of fucoidanase substrate specificity.

In this paper, we report the cloning of fucoidanase FFA2 from the marine bacteria *F. algeae* KMM 3553T, characterization of the catalytic properties of this enzyme and determination of its substrate specificity. Substrate specificity was assessed with the use of a series of natural fucoidans and synthetic oligosaccharide substrates.

### Results

#### Expression of the fucoidanase genes from *F. algeae*

KMM 3553T

Genome of fucoidanase producing bacterium *F. algeae* KMM 3553T was sequenced, and amino acid sequence of the gene encoding fucoidanase, referred to as *ffa2*, was obtained. The mass of the gene product FFA2 is 101.2 kDa (918 amino acid residues). Recombinant product of the gene *ffa2* was obtained to confirm its predicted function. Plasmid pet-22b(+) was used as the expression vector. The construct encoded the FFA2 (V37-N918) without of predicted signal sequence was obtained. Construct contained a nucleotide sequence encoding a C-terminal poly-histidine tag (6× His). Several commercial available strains of *Escherichia coli* were tested for efficient expression of fucoidanase (BL21 star (DE3)pLysS, Rosetta-gami (DE3)pLySS, BL21 (DE3)GroEL and Arctic Express (DE3)). The best strains of *E. coli* for producing recombinant fucoidanases were strains BL21 star (DE3)pLySS and Arctic Express (DE3).

**Substrate specificity of FFA2**

FFA2 catalyzed hydrolysis of the fucoidan from *F. evanescens* but not the fucoidans from *Saccharina cichorioides* and *Undaria*...
Similar data were obtained earlier in the experiments with native fucoidanase from *F. algae* (Silchenko et al. 2013). The products of hydrolysis of the fucoidan from *F. evanescens* were separated into two fractions, high-molecular-weight products (HMPs) and low-molecular-weight products (LMPs), by precipitation with 75% aqueous ethanol (Figure 3). The yields of LMP and HMP were 55 and 45%, respectively. It should be noted that elongation of the period of incubation of HMP with enzyme FFA2 or increasing in the concentration of FFA2 did not lead to deeper degradation of the polymer (Figure 2E). LMP was further fractionated by anion-exchange chromatography on DEAE-MacpoPrep to give compound 1 (Supplementary data, Fig. S1).

The structure of the derivatives HMP and 1 was investigated by NMR spectroscopy using 1D and 2D techniques (¹H, ¹³C, COSY, TOCSY, HSQC, HMBC). HSQC spectrum of HMP revealed the presence of two types of the dominant constituent L-fucopyranosyl residues A and B, as only two correlation peaks were observed in the anomeric region (Supplementary data, Fig. S2). α-Configuration of the glycoside bonds was confirmed by the characteristic value of ω₁,₂ constants of 3.4–3.5 Hz. HMBC spectrum of HMP showed that the fucose residue A was linked to O-3 of the residue B (correlation peak H-1(A)/C-3(B) at 5.39/74.0 ppm) and the residue B was linked to O-4 of the residue A (correlation peak H-1(B)/C-4(A) at 5.35/83.5 ppm) (Supplementary data, Fig. S3). Pattern of sulfation was easily determined using COSY spectrum (Supplementary data, Fig. S4). Thus, the downfield shift of H-2 (δ 4.46 ppm) indicated the residue A as Fuc2S, while the unit B was specified as Fuc2S₄S (δ H-2 4.55 ppm, H-4 4.95 ppm). The degree of polymerization was estimated by comparing the intensity of anomeric proton signals of residues A, B and reduced ends in the ¹H NMR spectrum and amounted to near 40 monosaccharide residues. The structure of HMP is shown in Figure 3 while its NMR chemical shifts are summarized in Table I.

HSQC spectrum of compound 1 contains four correlation peaks in the anomeric region, corresponding to four types of L-fucopyranoside residues A', C, A'' and D (Supplementary data, Fig. S5). Three correlation peaks in HMBC spectrum revealed the types of linkages between fucose residues: H-1(A')/C-3(C) at 5.35/73.5 ppm, H-1(C)/C-4(A'') at 5.29/83.8 ppm and H-1(A'')/C-3(D) at 5.34/73.9 ppm (Supplementary data, Fig. S6). Analysis of the COSY spectrum showed the presence of sulfate groups at O-2 of all fucosyl residues because of characteristic low-field location of corresponding H-2 signals (δ H-2 is 4.47 ppm for A', 4.60 ppm for C, 4.49 ppm for A'' and 4.52 ppm for D) (Supplementary data, Fig. S7). Thus, compound 1 was specified as the linear tetrasaccharide Fuc2S₁→3Fuc2S₁→4Fuc2S₁→3Fuc2S. Its structure is shown in Figure 3 while chemical shifts are presented in Table I.
The kinetics of hydrolysis of the fucoidan from *F. evanescens* by the recombinant fucoidanase FFA2 were analyzed by size-exclusion chromatography (SEC), carbohydrate-polyacrylamide gel electrophoresis (C-PAGE) and thin-layer chromatography (TLC). FFA2 catalyzes the formation of poly- and oligosaccharides with different degree of polymerization on the early stages of the enzymatic reaction (Figure 4). The LMPs (3.8–4.6 kDa) were detected after 45 min of incubation with FFA2 (Figure 4A). C-PAGE analysis revealed that a broad number of sulfated oligosaccharides were formed after several minutes of the reaction start. Elongation of the reaction time led to the decreasing of the degree of polymerization of the fucoidan derived products. Tetra- and disaccharides were formed after 15 min and 3 h of the reaction, respectively (Figure 4B and C).

To map the substrate specificity of FFA2 precisely, its effect on synthetic oligosaccharide substrates \(^{2,3,6,8,10,11}\) was studied next (Figure 5). These compounds are related to structural fragments of the fucoidan from *F. evanescens*. The result of enzymatic treatment was monitored by TLC (Figure 6). Thus, (1→4)-linked disaccharide \(^1\) remained intact. On the contrary, the synthetic tetrasaccharide \(^3\) was transformed during 24 h into equimolar mixture of mono- (5) and trisaccharide 4 (Figure 6). These products were identified by using of NMR spectroscopy (Supplementary data, Fig. S8).

**Table I.** The data of \(^1\)H and \(^1\)C NMR spectra of HMP and compound 1

<table>
<thead>
<tr>
<th>Residue (Figure 3)</th>
<th>(^1)H/(^1)C chemical shifts (ppm)</th>
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<tbody>
<tr>
<td></td>
<td>H-1/C-1</td>
</tr>
<tr>
<td>HMP</td>
<td></td>
</tr>
<tr>
<td>A→4)-α-L-Fucp2S-(1→</td>
<td>5.39/98.6</td>
</tr>
<tr>
<td>B→3)-α-L-Fucp2S,4S-(1→</td>
<td>5.35/99.6</td>
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<td>1</td>
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<tr>
<td>Aα-L-Fucp2S-(1→</td>
<td>5.35/95.0</td>
</tr>
<tr>
<td>C→3)-α-L-Fucp2S-(1→</td>
<td>5.29/100.6</td>
</tr>
<tr>
<td>A′→4)-α-L-Fucp2S-(1→</td>
<td>5.34/95.3</td>
</tr>
<tr>
<td>D→3)-α-L-Fucp2S</td>
<td>5.49/91.8</td>
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Fig. 4. Kinetics of hydrolysis of the fucoidan from *F. evanescens* by the recombinant fucoidanase FFA2. SEC (A), C-PAGE (B) and TLC (C) analyses of the products of hydrolysis of fucoidan from *F. evanescens* by FFA2; the numbers at the top of plots indicate the duration of enzymatic hydrolysis (in min or h). RID, refractometric index. Used control substances: fucoidan from *F. evanescence* (Cs F.e.); fucoidan from *F. evanescence* with addition of boiled FFA2 (C0); tetrasaccharide 1 (Cs (1)); tetrasaccharide 1 hydrolyzed by the FFA2 and produced the disaccharide 7 (FFA2 (1)).
Three unsulfated, per-O-sulfated and selectively octakis-(2-O-sulfated) synthetic octasaccharides (6, 10, 11) built up of alternating (1→4)- and (1→3)-linked α-L-fucosyl residues were also studied as substrates of FFA2. Fucoidanase did not catalyze the hydrolysis of nonsulfated (10) and per-O-sulfated (11) octasaccharides, while the substrate 6 build up only of 2-O-sulfated α-L-fucosyl units was efficiently cleaved to form a complex mixture of mono- and oligosaccharides (Figure 5). It is noticeable that the rate of hydrolysis of octasaccharide 6 was significantly higher than that one of tetrasaccharide 3 (Figure 6A).

Tetrasaccharide 3 was cleaved by FFA2 used at low concentration of 0.01 mg/mL, while tetrasaccharides 1 and 8 were not transformed under such conditions. Increasing of the concentration of the enzyme to 0.05 mg/mL and elongation of incubation time to 72 h led to a cleavage of 1 and 8 to sulfated disaccharides 7 and 9 (Figures 5 and 6B).

**Discussion**

Analysis of the primary structure of FFA2 showed that the amino acid sequence of fucoidanase FcnA from the marine bacterium *M. fucanivorans* SW5T (GenBank CAI47003.1) has 57% of identity with FFA2. Amino acid sequences of other known fucoidanases Fda1 from *Alteromonas* sp. SN-1009 (GenBank AA00508.1) and Fda2 (GenBank AA00509.1) and SVI_0379 from *S. violacea* DSS12T (GenBank BAJ00350.1) showed lower amino acid sequence identity with FFA2 (Figure 7).

The signal sequence in FFA2, which is a feature of extracellular proteins, has been found. A cleavage site of predicted signal sequence is between A32 and Q33. Using InterProScan, we revealed a modular architecture of FFA2. Cadherin-like repeat domains were found in amino acid sequence of fucoidanase. These domains include following sequences: domain K1 (Pro408-Gly509) and domain К2 (Gly522-Val616). Similar repetitive cadherin-like domains were found in fucoidanase FcnA. C-terminal domains of FFA2 (Leu822-Arg889) are highly conserved. We have found all domains in amino acid sequences of FFA2, which were found in the structure of FcnA previously (Colin et al. 2006).

Previously, Colin et al. reported low identity of the amino acid sequences between FcnA, Fda1 and Fda2 but showed the presence of hydrophobic clusters that are common for all N-terminal area of these fucoidanases. Authors proposed that these fucoidanases constitute one family of GH107 (Colin et al. 2006). We would like to...
point out that although these fucoidanases assigned to the same structural family they have different specificity of action. Fucoidanase FcnA is specific to α-(1→4)-glycoside bonds between sulfated fucose residues while Fda1 and Fda2 are capable to cleave fucoidan from brown algae *Kjellmaniella crassifolia* containing only α-(1→3)-linked fucose residues (Takayama et al. 2002). Comparison of amino acid sequence of the FFA2 with known fucoidanases revealed several conserved regions at N-terminal area (Figure 7). Probably these conserved sites are essential for all fucoidanases of this structural family (GH107). Thus, FFA2 belongs to glycoside hydro-lases GH107 family. Unfortunately, only a few data about structure and catalytic properties of the fucoidanases which are known to date do not suggest the possible function performed by these conserved regions: structural, catalytic, substrate-binding or other. So, besides FFA2, there is only one fucoidanase FcnA of the known amino acid sequences specific to α-(1→4)-glycoside linkages. Fucoidanases are known to bind specifically to certain structural fragments of fucoidans (Silchenko et al. 2013). Fucoidanase FFA2 was shown to hydrolyze the fucoidan from *F. evanescens*, which is built up of alternating (1→3)-α- and (1→4)-α-linked residues of sulfated fucose units. However, the enzyme did not effect on the fucoidans from *S. cichorioides* (sulfated homo-(1→3)-α-L-fucan (Vishchuk et al. 2013)) and *U. pinnatifida* (sulfated galactofucan containing only (1→3)-α-linked sulfated fucose residues (Synytsya et al. 2010)). Fucoidanase FFA2 did not cleave the fucoidan from *F. evanescens*. Two fractions, HMP and LMP, were obtained as a result of this reaction. HMP fraction was resistant to further enzyme action (Figure 2E). Formation of HMP indicates that fucoidan chain consists of blocks with a regular structure built up of the repeating 2,4-di-O-sulfated-(1→3)- and 2-O-sulfated-(1→4)-α-linked fucose residues (Figure 3). Apparently, the presence of disulfated fucose residue makes (1→4)-α-linkages unavailable for enzymatic treatment. Detection of such blocks by usual methods of structural carbohydrate analysis is practically impossible. Thus, in the process of studying the enzyme specificity, the new information about the structure of fucoidan from *F. evanescens* was obtained. Analysis of NMR spectra of product 1 showed reducing of the amount of α-(1→4)-linked fucose residues compared to native fucoidan whereas amount of α-(1→3)-linked fucose residues was not changed. This indicates that FFA2 is specific to hydrolyzes of α-(1→4)-glycoside bonds between two 2-O-sulfated fucose residues but not between 2-O- and 2,4-O-disulfated fucose residues. Investigation of the kinetic of enzymatic hydrolysis of the fucoidan showed a number of oligosaccharides with different degree of polymerization which appeared in the initial stage of reaction. Thus, FFA2 exhibits the endo-type of action. Shortest oligosaccharide product of action of FFA2 on fucoidan was disaccharide 7 (Figure 5). It is different from the tetrasaccharide as minimal product being obtained with the use of extracellular enzyme from *M. fucanivorans* (Descamps et al. 2006). It is known (Davies et al. 1997) that the specificity of polysaccharide hydrolases is directly related to size of their active sites. Probably FFA2 and FcnA have different structure of active sites that causes their different substrate specificity. Further investigation of the substrate specificity of FFA2 has been performed using a series of synthetic fucooligosaccharides of definite structure (Figure 3). Obtained results show that this enzyme FFA2 cleaves α-(1→4)-glycoside linkages in oligosaccharides built 

![Fig. 7. Multiple alignment of the amino acid sequences of the fucoidanases from *F. algae* KMM 3553T (FFA2), *M. fucanivorans* SW5T (FcnA), (*Alteromonas*) sp. SN-1009 (Fda1, Fda2), *S. violacea* DSS12T (SVI_0379).](https://academic.oup.com/glycob/article-abstract/27/3/254/2749179/fig7)
up of 2-O-sulfated fucose residues and has a degree of polymerization more than 2. Unsulfated (10) or persulfated (11) oligosaccharides were not cleaved by FFA2. It means that 2-O-sulfated fucose residues are important for formation of the Michaelis complex. Noteworthy is that the reaction of FFA2 with tetrascaracchide 3 lead to formation of propyl 2-sulfo-α-L-fucopyranoside 5 whereas sulfated fucose was absent among the products of enzymatic reactions to show the resistance of second (1→4)-bond located in the “non-reducing” end. This observation shows that FFA2 attacks the tetrascaracchide substrate from the reducing end.

Notably, the rate of enzymatic hydrolysis depends on the degree of polymerization. The octascaracchide 6 was cleaved much faster than the tetrascaracchide 3. This feature is characteristic for polysaccharide hydrolyases: the reaction rate increases with increasing of degree of polymerization of substrate. The sequence of glycoside bonds has an influence on the rate of the reaction also. Thus, tetrascaracchide 1 and synthetic sulfated tetrascaracchide 8 with bonds sequence (1→3)-(1→4)-(1→3) reacted much slower than the tetrascaracchide 3 bearing bonds sequence (1→4)-(1→3)-(1→4).

Conclusion

The unique fucoidanase FFA2 from *F. algae* was expressed in *E. coli* for the first time. Sequence analysis revealed that FFA2 is a member of the GH107 family (CAZY (Lombard et al. 2014)). Detailed substrate specificity was studied using fucoidans from brown seaweeds as well as synthetic fucoidosaccharides of distinct structure. Fucoidanase FFA2 catalyses the cleavage of (1→4)-α-glycoside bonds in the fucoidan from *F. evanescentis* in the fragments (→3)-α-L-Fucp2S-(1→4)-α-L-Fucp2S-(1→3) but not in the fragments (→3)-α-L-Fucp2SAS-(1→4)-α-L-Fucp2S-(1→3) containing 2,4-di-O-sulfated fucos unit. Using synthetic oligosaccharides built from the alternative (1→4)- and (1→3)-linked α-L-Fucp2S fucose unit with degrees of polymerization of 2, 4 and 8, a difference in the rate of enzymatic reaction was demonstrated. Nonsulfated and persulfated synthetic oligosaccharides were not transformed by the enzyme. As a result, the recombinant fucoidanase FFA2 was specified as 2-O-sulfo-fucan endo-(1→4)-α-L-fucanase. This enzyme could be considered as a convenient tool for fucoidanase transformation to obtain more regular and easier characterized derivatives useful for research and practical applications. The formation of highly regular polysaccharide product HMP which is resistant to fucoidanase FFA2 shows the possibility of block-wise structures along the fucoidan chain.

Materials and methods

Fucoidans

Crude fucoidans from *S. cichorioides*, *F. evanescentis* and *U. pinnatifida* were obtained as described (Zvyagintseva et al. 1999). Parent fucoidans were then purified by ion-exchange chromatography (Kusaykin et al. 2006) and O-deacetylated (Chizhov et al. 1999).

General methods

The total carbohydrate amount was determined using the phenol-sulfuric acid method with α-fucose as the standard (Dubois et al. 1956). The protein concentration was determined by the Bradford method (Bradford 1976) with bovine serum albumin as a standard. Isolation and purification of genomic DNA were performed using the GenElute Bacterial Genomic DNA Kit (Sigma, St. Louis, MO) according to the manufacturer’s protocol.

Sequence analysis

The genome of *F. algae* KMM 3553^T* (GenBank: PRJNA299442) was analyzed to reveal the presence of sequences similar to known fucoidanases. Prediction of fucoidanase gene was carried out using pBLAST hits to *F. algae* protein databases through the RAST server (Rapid Annotation Subsystem Technology) (Aziz et al. 2008). Sequences of fucoidanases FcnA (GenBank: CAH70031.1), Fda1 (GenBank: AA000508.1), Fda2 (GenBank: AA000509.1) and SVL_0379 (GenBank: BAJ00350.1) were used for comparison. Gene of fucoidanase ffa2 (GenBank: WP_057784219.1) has been identified in genome of the marine bacteria *F. algae* KMM 3553^T*. The identity of amino acid sequences of the fucoidanases was performed using the Clustal Omega service (Sievers et al. 2011). A signal peptide was predicted with using SignalP v3.0 (Bendtsen et al. 2004). InterProScan V5 was used for protein domains search (Quevillon et al. 2005).

Construction and cloning of the expression vectors

Constructs were cloned using the restriction-free (RF) cloning strategy (Bond and Naus 2012). The genomic DNA of *F. algae* KMM 3553^T* was used as the template for amplification of fucoidanases genes. The construct for gene of the fucoidanase ffa2 was designed to harbor a C-terminal his-tag (vector encoded). The gene of fucoidanase was amplified in a regular polymerase chain reaction (PCR) with high fidelity polymerase (New England Biolabs), which produces a primer pair that, once annealed to the 5207–5297 region of pet-22b(+)(Novogene), is extended in a linear amplification reaction. Primer design and PCR conditions were carried out using service at http://www.rf-cloning.org/. The sequence of forward primer was 5'-AATTTTGTGAATTTAGAGAGGAATATACATAGTGGTCGAGATTCAGGAGTTACATATCTTGCCAGATTTGATTAGGCT-3' and reverse primer was 5'-CTTGTGACGGAGGCTCAGATTACGGCTTAAACACCTTTTAAAC-3', where underlined sequence is vector-specific primer and nonunderlined is gene-specific primer. After RF cloning, parental pet-22b(+) vector was eliminated by treating the reaction mixture with DpnI restriction enzyme (1 µL of 20 U/µL for 2 h at 37°C; New England Biolabs). Then, XL10-Gold ultra-competent cells (Stratagene) were transformed by RF products. Colony PCR screening was performed using T7 primers. Positive clones were confirmed by DNA sequencing.

Production of recombinant fucoidanase FFA2

The protein expression of cloned fucoidanase genes was performed using the *E. coli* Arctic Express (DE3) strain. Recombinant strain harboring the pet-22b(+)/ffa2 recombinant plasmid was cultivated on LB medium with ampicillin (100 µg/mL) in shaking incubator at 210 r.p.m. and 37°C for 12 h. Suspension of culture was inoculated in LB medium (1:100, v/v) containing ampicillin (final concentration 100 µg/mL) and growth at 210 r.p.m. at 31°C up to OD_{600} = 0.4–0.6, then temperature was decreased to 16°C and gene expression was induced by IPTG (final concentration 0.4 mM). After induction, transformed bacteria were cultured in a shaking incubator at 200 r.p.m. and 16°C for 48 h.

Purification of fucoidanase FFA2

All purification steps were performed at 4°C. Bacterial cells were collected by centrifugation for 30 min at 4500 × g. The 0.04 M Tris–HCl buffer pH 7.5 (with addition of 0.5 M NaCl and 0.01 M imidazole) was added to 5 g of the bacterial biomass in a 3:1 (v/w) ratio. This mixture was disrupted by sonication at 20 kHz, five times
for 5 min each. The suspension was centrifuged at 12,000 × g for 40 min to remove the cellular debris. The supernatant (13 mL) was subjected to a HisTrap column (5 mL, GE Healthcare) equilibrated with the 0.5 M NaCl and 0.01 M imidazole in 0.04 M Tris–HCl buffer pH 7.5. The proteins were eluted with a linear gradient of imidazole (from 0.02 to 0.3 M) in a buffer (0.04 M Tris–HCl buffer pH 7.5 with 0.5 M NaCl) volume of 50 mL and a flow rate 2 mL/min. The fractions with fucoidanase activity were desalted on imidazole (from 0.02 to 0.3 M) in a buffer (0.04 M Tris–HCl buffer pH 7.5. The proteins were eluted with a linear gradient of 10 μM to 250 μM imidazole (from 0.1 to 1 M) in a 0.02 Tris–HCl buffer pH 7.2 (total volume: 40 mL) and at flow rate 0.5 mL/min. The fractions with fucoidanase activity were concentrated by ultrafiltration through a 10-kDa cut-off membrane to a final volume of 2 mL and applied to a Sephacryl S-200 column (1 × 200 cm) equilibrated with 0.02 M Tris–HCl buffer pH 7.0. The proteins were eluted with the same buffer at a flow rate of 0.15 mL/min. The fractions with fucoidanase activity were pooled and stored at −20 °C.

**Sodium dodecyl sulfate-electrophoresis of proteins**

The homogeneity and molecular weight of proteins were estimated by sodium dodecyl sulfate–PAGE (SDS–PAGE) electrophoresis according to the Laemmli protocol (Laemmli 1970). Electrophoresis was performed in 12% acrylamide gels with the addition of detergent—SDS. The Protein Plus molecular weight marker (Bio-Rad) with molecular weights of 10–250 kDa was used as standard. Images of gels were obtained using a densitometer GS-800 (Bio-Rad). The molecular weights of proteins obtained by PAGE were calculated using the QuantityOne 4.6.7 program (Bio-Rad).

**Synthesis of fucooligosaccharides**

Disaccharide 2, tetrascarides 3, 8 and octascarides 6, 10 and 11 were synthesized as described previously (Ustyuzhanina et al. 2006, 2009; Krylov et al. 2011; Ustyuzhanina et al. 2014a, 2014b).

**Enzyme activity assay**

Fucoidanase FFA2 activity was monitored by C-PAGE and TLC as described below.

**C-PAGE analysis**

Fucoidanase activity was detected by the occurrence of charged oligosaccharide bands in the gel. The reaction mixture containing 10 μL of enzyme solution (0.01–0.1 mg/mL) in 0.02 M Tris–HCl buffer pH 7.0 with 5 mM CaCl2 (buffer A) and 10 μL of fucoidan (from *F. evanescens*, *U. pinnatifida* or *S. cichoroides*) solution (20 mg/mL in buffer A were incubated at 34°C for 30 min to 24 h. The reaction was stopped by heating at 80°C for 5 min. The hydrolysis products were mixed with 5 μL of loading buffer containing a 20% solution of glycerol in water and 0.02% phenol red. The samples (5 μL) were electrophoresed through a 5% (w/v) stacking gel with 50 mM Tris–HCl buffer pH 6.8 and 27% (w/v) resolving polyacrylamide gel with 150 mM Tris–HCl buffer pH 8.8. The gel was 1 mm thick. Gel staining was performed with a solution containing 0.02% O-toluidine blue (Sigma, St. Louis, MO) and 0.3% alcin blue in EtOH, AcOH and H2O with a volume ratio of 2:1:1 or with 0.05% alcin blue 8 GX (Panreac, Spain) in 2% acetic acid.

**TLC analysis**

The reaction mixture containing the enzyme solution (10 μL) at a concentration of 0.01 or 0.03 mg/mL in buffer A and 10 μL of fucoidan (20 mg/mL) or fucooligosaccharides (10 mg/mL) solutions in buffer A was stored at 34°C for 30 min. The reaction was stopped by heating at 90°C for 2 min. The hydrolysis products (2 μL) were loaded onto the TLC plate (Silica Gel 60F254, Merck) and eluted in the system AcOH:EtOH:H2O in a ratio 25:31:22 for sulfated and 2:2:1 for unsulfated fucooligosaccharides, respectively. Products of the enzymatic reaction were visualized by heating at 120°C after spraying with 10% (v/v) sulfuric acid in ethanol.

**Determination of the pH optimum for fucoidanase FFA2 activity**

The reaction mixture, containing 50 μL of fucoidanase FFA2 solution (0.01 mg/mL) in buffer A, 150 μg of *F. evanescens* fucoidan and 25 μL of buffers with various pH values (0.2 M citrate-phosphate buffers with pH values ranging from 3.0 to 8.5 or borate buffer pH 9.0), was incubated for 30 min at 34°C. Activity levels were monitored by C-PAGE as described above.

**Determination of the optimal temperature for fucoidanase FFA2 activity**

The fucoidanase FFA2 solutions (10 μL) in buffer A were incubated with 10 μL of *F. evanescens* fucoidan solution (20 mg/mL) at different temperatures (4, 20, 25, 30, 35, 37, 50 and 55°C) for 30 min. Enzyme activity was monitored as described above.

**Influence of ions of multivalent metals on fucoidanase FFA2 activity**

A reaction mixture containing enzyme solution (10 μL), 10 μL of *F. evanescens* fucoidan solution (20 mg/mL) and a solution (2 μL of 0.1 M) of the appropriate salt [(AlCl3), (BaCl2), (CaCl2), (CoCl2), (CuSO4), (FeCl3), (MgCl2), (MnCl2) or (SnCl2)] was incubated for 1 h at 34°C, and the activity level was determined by C-PAGE as described above.

**SEC analysis**

Samples were analyzed using a high-performance liquid chromatography instrument (Agilent 1100 Series, Germany) equipped with a refractive index detector and a series-connected gel-filtration column (TSK gel G4000 SW and TSK gel G2000 SW, Tosoh Co., Japan). Elution was performed with 0.05 M Na2SO4 aqueous solution at 50°C with a flow rate 0.5 mL/min. Molecular weight of the fucoidan and the products of its enzymatic hydrolysis was estimated using dextrans of molecular weights of 6, 10, 40 and 80 kDa (Sigma, St. Louis, MO) as reference standards.

**Kinetics of the enzymatic reaction**

The kinetics of fucoidan hydrolysis by fucoidanase FFA2 were evaluated by PAGE, TLC and SEC. The reaction mixture containing 300 μL of the fucoidanase solution in buffer A (0.05 mg/mL) and 300 μL of fucoidan solution in buffer A (20 mg/mL) was incubated at 34°C for 10, 15, 30, 90 min and 3, 9, 24, 48, 72 h. Aliquots 10 μL of the reaction mixture were taken for each of the methods (PAGE, TLC and SEC). The reaction was stopped by heating at 80°C for 5 min.
Preparation of enzymatic hydrolysis products

Fucoidan from *F. evanesens* (1 g) was dissolved in 98 mL of buffer A, and 2 mL of FFA2 (0.1 mg/mL) was added. The reaction mixture was incubated at 34°C for 72 h and then deproteinized by heating at 80°C for 10 min, precipitate was removed by centrifugation. The high-molecular-weight reaction products were precipitated with ethanol at the ratio of 1:3 (v/v) and precipitate was separated by centrifugation at 10,000 × g for 40 min. The supernatant containing low-molecular-weight reaction products was concentrated under vacuum. Then, LMPs were applied onto DEAE-Macroprep column (1 × 10 cm) equilibrated with water. Oligosaccharides were eluted with a linear gradient (from 0 to 1 M) of solution of NaCl in working buffer. Total volume was 300 mL and flow rate was 0.7 mL/min. The fractions containing the carbohydrates were pooled, concentrated by vacuum evaporation and desalted on Sephadex G-10 column. The carbohydrates in the fractions were detected with the phenol–sulfuric acid method (25).

Tetrasaccharide 3 (5 mg) was dissolved in 0.5 mL of buffer A and 0.1 mL of FFA2 (0.05 mg/mL) was added. The reaction mixture was incubated at 34°C for 24 h, deproteinized by heating at 80°C for 10 min and subjected to Sephadex G-15 column. The fractions contained carbohydrates were concentrated under vacuum and then studied by NMR spectroscopy.

NMR spectroscopy

NMR spectra were recorded using Avance DPX-500 NMR spectrometer (Bruker, Germany). 1H, 13C spectra and 2D spectra (COSY, TOCSY, HSQC, HMBC) were recorded for solutions of ploy- and oligosaccharides in D2O at 50°C with acetone as the internal standard. The concentration of the samples was 5–20 mg/mL.

**Supplementary data**

Supplementary data for this article is available at *Glycobiology* online.

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**Abbreviations**

C-PAGE, carbohydrate-polyacrylamide gel electrophoresis; DP, degree of polymerization; HMP, high-molecular-weight product; LMP, low-molecular-weight product; RID, refractometric index; SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography; TLC, thin-layer chromatography.

**References**


