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Heterologous expression and functional characterization of the homologous fucoidanase from *Formosa haliotis*

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ABSTRACT

Fucoidanases, enzymes that catalyze the degradation of fucoidan—a sulfated polysaccharide from brown algae—have garnered increasing attention for their potential in biomedical, pharmaceutical, and food applications. In this study, we report the heterologous expression and functional characterization of Fhf3ΔSPNW, a homologous fucoidanase derived from the marine bacterium *Formosa haliotis*. A truncated version of the gene was amplified and successfully cloned into the pET31b(+) vector, followed by transformation into *Escherichia coli* BL21(DE3) for protein expression. The recombinant protein, with a molecular weight of ~52 kDa, was confirmed using SDS-PAGE and Western blot analysis. Purification using Ni-NTA affinity chromatography yielded high-purity enzyme fractions. Functional assays demonstrated that *Fhf3ΔSPNW* exhibited specific activity against fucoidan from brown seaweed *Fucus evanescens*, suggesting a preference for substrates with alternating α(1→3)/α(1→4) linkages and moderate sulfation. These findings highlight *Fhf3ΔSPNW* as a promising candidate for the selective production of bioactive oligo-fucoidans, and contribute to the growing toolbox of homologous fucoidanases for tailored enzymatic applications.

Keywords: Homologous fucoidanase, GH107, substrate specificity, *Formosa haliotis*, oligo-fucoidan.

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INTRODUCTION

Fucoidanases are enzymes capable of hydrolyzing fucoidan - sulfated polysaccharides primarily found in the cell walls of brown seaweeds - into bioactive oligosaccharides [1, 2]. These enzymes have recently emerged as a valuable tool for overcoming the complex and heterogeneous structure of fucoidan, which poses challenges for its characterization and biotechnological utilization [3, 4]. By enabling the selective cleavage of glycosidic bonds, fucoidanases support both structural elucidation and the production of low-molecular-weight derivatives with enhanced biological activities [5–7]. Among various sources, marine bacteria have proven to be a rich reservoir of fucoidanases with high substrate specificity and catalytic efficiency, holding strong potential for applications in biomedicine, nutraceuticals, and marine biotechnology [6, 8, 9].

The marine environment, with its extreme salinity, variable temperatures, and diverse ecological niches, exerts unique selective pressures that drive microbial adaptation and enzymatic specialization. As a result, marine-derived fucoidanases often possess superior stability, salt tolerance, and substrate selectivity, making them particularly attractive for industrial and biomedical applications [10, 11]. Several fucoidan-degrading bacteria have been isolated from marine ecosystems, including *Formosa algae* [12], *Wenyingzhuangia fucanilytica* [13], *Vibrio* sp. N-5 [14], *Sphingomonas paucimobilis* PF-1 [15], and *Luteolibacter algae* [16], each producing distinct fucoidanases with unique structural and functional characteristics. These microbial sources not only reflect the enzymatic diversity shaped by marine conditions but also serve as valuable platforms for the discovery of novel fucoidanases with tailored properties for specific biotechnological needs.

Among various fucoidanases reported to date, homologous fucoidanases, enzymes that share a common evolutionary origin and exhibit structural similarities, often display diverse substrate specificities and catalytic properties [9, 13, 17]. For instance, the two fucoidanases FFA1 and FFA2, both isolated from *F. algae*, share high sequence similarity yet exhibit distinct biochemical characteristics, including differences in substrate recognition and cleavage specificity [12, 18]. Similarly, several homologous enzymes, FWf1–FWf4, identified in *W. fucanilytica* display notable variations in catalytic efficiency, optimal pH, and degradation profiles, despite their phylogenetic relatedness [13]. These findings highlight the functional divergence that can arise among homologous fucoidanases, underscoring the structural complexity of fucoidan and the enzymatic versatility required for its selective depolymerization [9].

In line with these observations, two homologous fucoidanases, Fhf1 and Fhf2, have been previously identified from the marine bacterium *F. haliotis*. Both enzymes specifically cleave α-1,4-fucosidic linkages in the fucoidan backbone and are classifed into the GH107 fucoidanase family. Even though they exhibit distinct substrate preferences and hydrolytic profiles. Fhf1 demonstrates higher activity on fucoidan from *Fucus evanescens*, whereas Fhf2 displays a broader substrate range, acting on fucoidans from additional Vietnam brown algae Sargassum species [9, 17]. These differences highlight functional divergence despite their structural similarities. The oligosaccharides released by each enzyme differ in polymerization degree and sulfation pattern, which can influence their bioactive properties. These variations emphasize the need for further exploration of fucoidanases, as the discovery of new homologous enzymes could offer a wider range of bioactive oligo-fucoidans with diverse therapeutic potentials [5, 19].

To further explore the enzymatic diversity within this species, we have identified a third putative GH107 fucoidanase gene, designated *fhf3*, in the genome of *F. haliotis*. The enzymatic function of this gene product remains uncharacterized. Therefore, in this preliminary study, we aim to clone, express, and purify *Fhf3ΔSPNW* fucoidanase, and to conduct an initial investigation into its substrate specificity. These efforts are intended to provide the groundwork for understanding the functional variation among homologous fucoidanases and to offer new insights into their potential utility in fucoidan degradation.

MATERIALS AND METHODS

Materials and media composition

Escherichia coli DH5α (Invitrogen, Waltham, MA, USA) was used as the host for plasmid clones. *E. coli* BL21 (DE3) harboring the Pch2 (pGro7) plasmid (Takara, Goteborg, Sweden) was used for protein expression.

Plasmid pET31b(+) (Thermo Fisher Scientific, Waltham, MA, United States) was used for fucoidanase expression under the control of the T7-promoter induced by isopropyl-β-D-1 thiogalactopyranoside (IPTG) (Sigma Aldrich, USA).

Bacterial strain were propagated using LB medium (Luria-Bertani) containing 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl. For LB agar, 2% (w/v) agar was added, pH adjusted to 7.0–7.5. Ampicillin (100 µg/mL) and chloramphenicol (35 µg/ml) were added as required.

Fhf3ΔSPNW Gene Cloning

The *fhf3* gene from *F. haliotis*, lacking the predicted N-terminal predicted signal peptide and containing a C-terminal 6xHis-tag was synthesized codon-optimized for *E. coli* expression and subcloned into the pET31b(+) vector between the NdeI and XhoI restriction sites (Thermo Fisher Scientific, Waltham, MA, USA). A C-terminally truncated version, *Fhf3ΔSPNW*, was obtained by removing the last 404 amino acids corresponding to the two predicted CBMs. A C-terminal 10xHis-tag was then added.

The *fhf3* gene was used as the template. The *Fhf3ΔSPNW* gene was amplified using the following pair of primers: Fhf3ΔSPNW_F 5'-CATATGCAACAAATACCCGATCCAG-3' and Fhf3ΔSPNW_R 5'-CAGTCATCTCGAGCTAATGGT GATGGTGATGGTGCGGCGCACCCGGATATTGGTT AAC-3', as forward and reverse primer, respectively. PCR was carried out in a 25 µL reaction containing: 1 µL plasmid DNA (500 ng), 12.5 µL CloneAmp HiFi polymerase premix (Takara Bio, CA, USA), 1 µL of each 10 µM primer, and 9.5 µL distilled water. Thermal cycling conditions were as follows: 30 s pre-denaturation at 98°C; 20 cycles of 10 s at 98°C, 10 s at 58°C, and 60 s at 72°C; followed by a 10 min final extension at 72°C. PCR products were treated with DpnI at 37°C overnight and purified using the GFX™ PCR DNA Purification Kit (GE Healthcare, Uppsala, Sweden). Amplified products were verified by 1% (w/v) agarose gel electrophoresis.

The purified PCR product and pET31b(+) vector was digested with NdeI and XhoI for 4 h at 37°C and ligated using T4 DNA ligase (Thermo Fisher Scientific, Waltham, MA, United States) at a 3:1 insert-to-vector mass ratio at 16°C overnight. The ligation product was used to transform *E. coli* DH5α as plasmid propagation host (Invitrogen® Life Technologies, MA, USA). Positive transformants were selected on LB agar plates containing 100 µg/mL ampicillin. Recombinant plasmids, pET31b(+)_fhf3ΔSPNW, were extracted and confirmed by DNA sequencing (Macrogen Europe, Amsterdam, Netherlands).

Expression and Purification of *Fhf3ΔSPNW* Fucoidanase

The plasmids pET31b(+)_Fhf3ΔSPNW was transformed into *E. coli* BL21 (DE3) harboring the Pch2 (pGro7) plasmid. Positive transformed cells were selected on LB agar plates containing 100 µg/mL ampicillin and 35 µg/mL chloramphenicol. A single colony was inoculated into 10 mL LB broth and incubated overnight at 37°C, 200 rpm. Then, 5 mL of this pre-culture were transferred to 500 mL of LB medium in a 1 L shake flask. Cells were grown at 37°C until OD_{600} = 0.6–0.8, then IPTG was added to a final concentration of 1 mM to induce expression, followed by incubation at 20°C, 200 rpm for 18 h. Cells were harvested by centrifugation at 8,000 rpm for 30 min at 4°C.

In order to retain activity of the recombinant protein, the purification was conducted on ice. Cell pellets were resuspended in binding buffer (20 mM Tris-HCl, 250 mM NaCl, 10 mM Imidazole, pH 7.4) and lysed by sonication (10 cycles of alternate 10 s ON and 20 s OFF of pulses at 50% amplitude of UP 400S ultrasonic sonicator). The suspension was centrifuged at 15,000 rpm for 20 min. The

supernatant containing the soluble recombinant protein was collected and filtered through filter of 0.22 µm. The supernatant containing crude enzyme was loaded into Ni-NTA column with a volume of 1 mL resin (GE Healthcare, Chicago, IL, USA) pre-equilibrated in binding buffer. The column was washed 3 times with 1 mL wash buffer (20 mM Tris-HCl, 250 mM NaCl, 20 mM Imidazole, pH 7.4). The recombinant proteins were eluted with 1 mL/fraction by elution buffer with 100, 150, and 200 mM imidazole. The fractions were desalted on PD10 columns thereby removing imidazole (GE Healthcare, Chicago, IL, USA) according to manufactory's recommendations. All protein fractions were stored at -80°C for further analysis.

Samples from each step were analyzed by sodium dodecyl sulfate-polysaccharide gel electrophoresis assay (SDS-PAGE) and Western blotting using monoclonal anti-polyHistidine peroxidase-conjugated antibodies (Sigma-Aldrich, Steinheim, Germany). Protein markers were Precision Plus Protein standard for SDS-PAGE, Precision Plus Protein Dual Color Standard for Western Blots (Bio-Rad, Hercules, CA, USA). Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard [20].

Enzymatic assays and Carbohydrate polyacrylamide gel electrophoresis (C-PAGE)

Fucoidanase activity was evaluated using a reaction mixture containing 0.9% (w/v) fucoidan from five brown seaweed (*Fucus evanescens, F. vesiculosus, Sargassum mcclurei, S. polycystum*, and *T. ornate*) as substrate, 0.5 mg/mL of enzyme, 20 mM Tris-HCl (pH 7.4–8.0), 100–125 mM NaCl, and 10 mM $CaCl_2$. Reactions were incubated at 30°C for 18 h, and terminated by heating at 80°C for 5 minutes.

Reaction products were subsequently analyzed using C-PAGE as previously described in [17]. The oligosaccharide standard (St) was prepared by enzymatic digestion of 1% (w/v) *F. evanescens* fucoidan using 0.3 mg/mL FFA2 from *F. algae*. The lowest band, observed in the similarly treated standard derived from deacetylated *F. evanescens* fucoidan, corresponded to a tetrasaccharide composed of (1→4)- and (1→3)-linked α-L-fucosyl residues, each fucosyl unit sulfated at C2, as described by Silchenko et al., (2017) [18].

RESULTS AND DISCUSSION

Construction and Transformation of Recombinant Vector pET31b(+)_Fhf3ΔSPNW into *E. coli* DH5α and BL21(DE3)

To facilitate heterologous expression of the fucoidanase gene *Fhf3ΔSPNW*, the gene fragment was amplified via PCR using specific primers (Fhf3-F and Fhf3-R). The resulting amplicon was approximately 1377 bp in length, consistent with the expected size of the truncated gene (Fig. 1A, Lane 2), confirming successful amplification. No amplification product was observed in the negative control (Lane 1), further validating the specificity of the reaction.

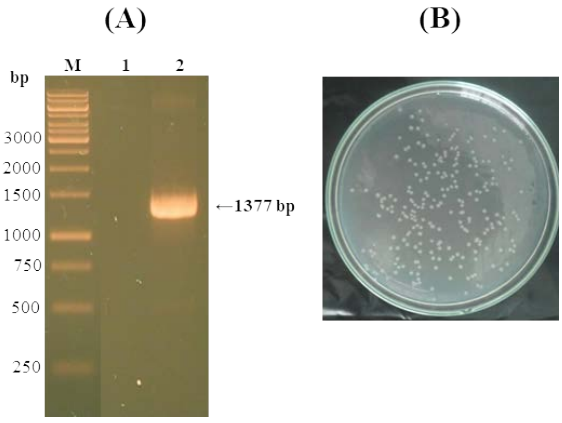

Figure 1. Agarose gel electrophoresis of *Fhf3ΔSPNW* gene (A) and colonies of *E. coli* DH5α strain with recombinant plasmid pET31b(+)_Fhf3ΔSPNW on LB agar with 100 µg/mL ampicillin (B). M - 1 kb DNA size ladder; 1- negative sample; 2- *Fhf3ΔSPNW* gene PCR product

The purified PCR product and the pET31b(+) expression vector were double-digested with NdeI and XhoI, followed by ligation using T4 DNA ligase. The ligation product was transformed into *E. coli* DH5α for plasmid propagation and

selection. White colonies appearing on LB agar containing 100 µg/mL ampicillin were screened for positive clones (Fig. 1B). Plasmids extracted from these colonies were sequenced using T7 primers (sequence IDs: 1CAFZAD-036, 1CAFZAD-037). Sequence alignment showed 100% identity to the designed insert, including the correct translational reading frame, confirming the accurate construction of pET31b(+)_Fhf3ΔSPNW (data not shown). Subsequently, the confirmed recombinant plasmid was introduced into *E. coli* BL21(DE3), a commonly used host strain for T7 promoter-driven protein expression. Colony PCR of transformants showed a clear 1377 bp band on agarose gel, aligning with the positive clone pattern (Figure 1A, Lane 2), thus confirming successful transformation into the expression host.

These results are consistent with standard molecular cloning approaches employed in other fucoidanase studies. For instance, Fhf1Δ470 and Fhf2Δ484 from *F. haliotis* were similarly cloned into pET-based expression vectors and transformed into *E. coli* BL21(DE3) for expression studies [9, 17]. Likewise, a recent study reported the cloning of fucoidanase genes FWf1–FWf4 from *W. fucanilytica* into pET-28a(+), which also utilized *E. coli* DH5α for plasmid propagation and BL21(DE3) for expression [13]. Compared to these previous constructs, the use of pET31b(+) vector, which encodes a C-terminal His-tag fusion, may enhance downstream purification efficiency.

Collectively, these findings establish a solid molecular foundation for the production of recombinant Fhf3ΔSPNW, enabling its biochemical characterization and comparative functional analyses.

Expression and Purification of Recombinant *Fhf3ΔSPNW* Fucoidanase

To evaluate the biochemical characteristics of the fucoidanase *Fhf3ΔSPNW*, the recombinant protein was heterologously expressed in *Escherichia coli* BL21(DE3) carrying the plasmid vector encoding the gene of interest. Following induction with 1 mM IPTG, the cells were harvested and disrupted by ultrasonication, consistent with the intracellular nature of most fucoidanases. The crude extract obtained after centrifugation and filtration was subjected to purification using Ni^{2+}-affinity chromatography, exploiting the His-tag fusion of the recombinant protein.

SDS-PAGE analysis (Fig. 2A) revealed a prominent protein band at approximately 52 kDa in the lysate of induced cells (Lane 1), aligning with the predicted molecular weight of *Fhf3ΔSPNW*. No corresponding band was observed in the uninduced control (data not shown), confirming successful expression. Western blot analysis (Fig. 2B) further validated the identity of this band as *Fhf3ΔSPNW*, with strong immunoreactivity observed at the same molecular weight (Lane 1). In contrast, proteins present in the wash fraction (Lane 2) appeared on SDS-PAGE but showed no signal in Western blot, indicating that nonspecific proteins were efficiently removed, while the target enzyme remained bound to the resin. Elution of the bound protein was carried out using buffers containing 100, 150, and 200 mM imidazole. The majority of the target enzyme was recovered in the 100 and 150 mM fractions (Lanes 3 and 4), as evidenced by the presence of a single band at 52 kDa in both SDS-PAGE and Western blot. The 200 mM fraction (Lane 5) did not show any target band, suggesting that complete elution was achieved at lower imidazole concentrations. These results demonstrate the efficiency and specificity of the purification protocol for obtaining high-purity recombinant Fhf3ΔSPNW.

These findings are in line with previous studies on homologous enzymes Fhf1Δ470 and Fhf2Δ484, also derived from *Formosa haliotis*, which were similarly expressed in *E. coli* and purified using Ni-NTA affinity chromatography, with optimal elution at 100–150 mM imidazole [9, 17]. All three enzymes belong to the GH107 family and display comparable molecular weights: *Fhf3ΔSPNW* (~52 kDa), Fhf1Δ470 (~54 kDa), and Fhf2Δ484 (~50 kDa), supporting their structural relatedness.

In a related study, the novel enzyme OUC-FaFcn1 from *Flavobacterium algicola* was successfully produced in *E. coli* and purified via Ni-NTA chromatography [21]. Although its

molecular weight was not specified, the enzyme showed optimal activity at 40°C and pH 9.0, effectively degrading fucoidan into disaccharides. This highlights the diversity in functional properties among fucoidanases from marine bacteria. A broader review of fucoidanases has shown that these enzymes vary widely in source (bacteria, fungi, mollusks), molecular weight, and purification strategy [3, 5]. Ni-NTA affinity chromatography, DEAE-cellulose ion-exchange, and gel filtration are commonly employed. Activity is strongly influenced by pH, temperature, and metal ions, though purification consistency and yield remain a challenge for many systems. Compared to other reports, Fhf3ΔSPNW demonstrated high expression yield and purification clarity, suggesting enhanced processability. Its sharp band profile and lack of contaminant signal in higher imidazole fractions indicate a favorable expression–purification profile relative to its homologs. This provides a solid foundation for downstream characterization of its enzymatic activity and substrate selectivity, further explored in subsequent sections.

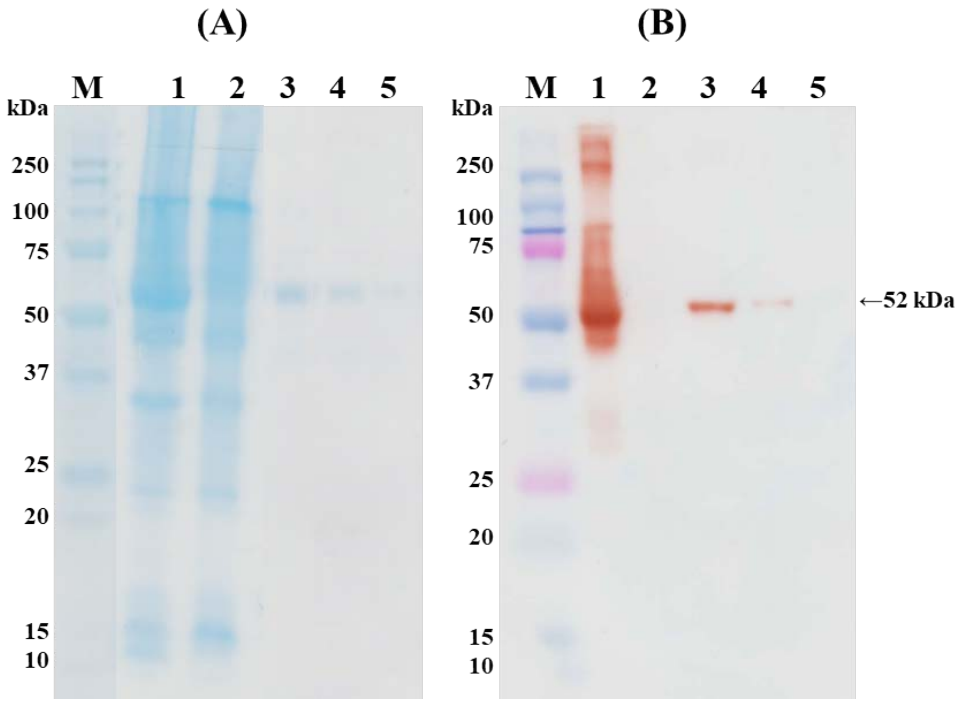

Figure 2. Expression and purification analysis of recombinant *Fhf3ΔSPNW* by SDS-PAGE (A) and Western blotting (B). M- protein marker; 1- induced cell; 2- wash solution; 3, 4, 5- purified fractions at 100, 150 and 200 mM Imidazole, respectively

Enzymatic Activity of *Fhf3ΔSPNW* Fucoidanase

To further elucidate the functional characteristics of *Fhf3ΔSPNW*, its enzymatic activity was assessed against fucoidans extracted from five brown seaweed species: *F. evanescens, F. vesiculosus, S. mcclurei, S. polycystum*, and *T. ornata*. Distinct differences in degradation profiles were observed, thereby revealing key aspects of the enzyme's substrate specificity and catalytic efficiency (Fig. 3).

The standard oliogosaccharide lane (St) provided a reliable reference for evaluating the extent of fucoidan hydrolysis. In the control lanes (-) for all tested species, no degradation bands were detected, confirming the stability of the substrates in the absence of the absence of anzymatic action. In contrast, the enzyme

treated lanes (+), displayed clear degradation patterns for *F. evanescens* (Fe) and *F. vesiculosus* (Fv), indicating that Fhf3ΔSPNW actively hydrolyzes fucoidans derived from these species. However, no visible degradation was observed for fucoidan from *S. mcclurei* (Sm), *S. polycystum* (Sp), and *T. ornata* (To), suggesting minimal to no enzymatic activity on these structurally distinct substrates (Fig. 3).

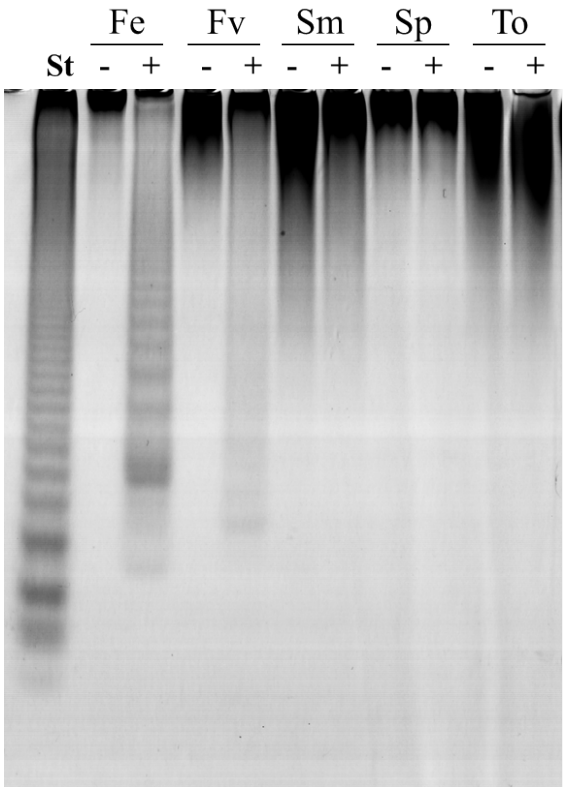

Figure 3. The activity of *Fhf3ΔSPNW* on fucoidan from brown seaweeds by C-PAGE. St-oligosaccharide standard; (-) control substrate without enzyme addition; (+) enzyme reaction of *Fhf3ΔSPNW* on fucoidan derived from various brown seaweed species: *F. evanescens* (Fe), *F. vesiculosus* (Fv), *S. mcclurei* (Sm), *S.polycystum* (Sp), and *T. ornata* (To)

Fhf3ΔSPNW demonstrated the highest activity on fucoidan from *F. evanescens*, which is known to comprise a regular backbone of alternating α(1→3) and α(1→4) linked fucosyl residues with primarily sulfated at the C2 position. Comparatively lower activity was observed on *F. vesiculosus* fucoidan, which although also composed of alternating α(1→3) and α(1→4) linkages, exhibits a higher degree of sulfation, including 2,3- and 2,4-di-O-sulfated fucosyl residues. These additional sulfate groups may cause steric hindrance or electrostatic repulsion, reducing substrate affinity and cleavage efficiency. In contrast, no enzymatic degradation was detected for fucoidans from *S. mcclurei* and S*. polycystum*, which are predominantly galactofucans with highly branched structures and a diverse composition of fucose, galactose, and occasionally uronic acids [17, 18]. Similarly, the linear α(1→3)-linked fucoidan from *T. ornata*, which lacks α(1→4) linkages, was resistant to hydrolysis. These findings suggest that Fhf3ΔSPNW requires the presence of alternating α(1→3) and α(1→4) linkages for catalytic activity, and its activity is markedly reduced by extensive branching or the absence of α(1→4) bonds.

These substrate preferences are consistent with those reported for homologous enzymes Fhf1Δ470 and Fhf2Δ484, also isolated from *F. haliotis*, which exhibited similar patterns of activity—strong on *F. evanescens*, moderate on *F. vesiculosus*, and negligible on Sargassum or Turbinaria-derived fucoidans [9, 17]. However, Fhf3ΔSPNW displayed a sharper distinction in substrate selectivity, particularly in its lack of detectable activity on complex galactofucans, indicating a potentially narrower substrate range compared to its homologs. This suggests functional specialization among homologous fucoidanases and provides insight into the evolutionary diversification of fucoidanases within marine bacteria.

Together, these results underscore the high specificity of Fhf3ΔSPNW for fucoidans with a regular, alternating α(1→3)/α(1→4) glycosidic backbone and moderate sulfation. The enzyme exhibits no activity toward heavily sulfated, highly branched, or uniformly α(1→3)-linked fucoidans. This selective substrate preference highlights its potential as a biocatalyst for structural characterization or tailored degradation of specific fucoidan types.

CONCLUSION

This study reports the successful cloning, expression, and purification of *Fhf3ΔSPNW*, a novel GH107 homologous fucoidanase from *Formosa haliotis*, expanding the known enzyme set alongside Fhf1 and Fhf2. While all three enzymes hydrolyze α-1,4-L-fucosidic linkages, *Fhf3ΔSPNW* exhibits a distinct substrate preference for moderately sulfated fucoidans with alternating α(1→3)/α(1→4) linkages, and lacks activity on highly sulfated or branched galactofucans. These findings highlight the functional divergence among homologous fucoidanases and reinforce the need to discover and characterize new variants with tailored substrate specificities. Although the recombinant enzyme was obtained with high purity, the current purification strategy has not yet been optimized for yield or scalability. The catalytic mechanism, kinetic parameters, and functional performance under industrially relevant conditions remain to be elucidated. Future work should focus on optimizing purification and scaling up enzymatic reactions to generate sufficient quantities of low-molecular-weight fucoidan products for downstream functional studies.

The identification of additional homologous enzymes with distinct catalytic properties will further enrich the enzymatic toolkit for fucoidan modification and contribute to the development of bioactive oligosaccharides for applications in biomedicine, food, and cosmetics. This work provides not only new insights into the enzymatic diversity of *F. haliotis*, but also a meaningful foundation for advancing fucoidanase research in Vietnam.

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