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Ohmes, Julia; Mikkelsen, Maria Dalgaard; Thi, Thuan Nguyen; Ha, Vy Tran Nguyen; Meier, Sebastian; Nielsen, Mads Suhr; Ding, Ming; Seekamp, Andreas; Meyer, Anne S.; Fuchs, Sabine

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Depolymerization of Fucoidan with Endo-Fucoidanase Changes Bioactivity in Processes Relevant for Bone Regeneration

Julia Ohmes a, Maria Dalgaard Mikkelsen b, Thuan Thi Nguyen bc, Vy Ha Nguyen Tran bc, Sebastian Meier d, Mads Suhr Nielsen e, Ming Ding e, Andreas Seekamp a, Anne S. Meyer b, and Sabine Fuchs a,*

a Experimental Trauma Surgery, Department of Orthopedics and Trauma Surgery, University Medical Center Schleswig-Holstein, 24105 Kiel, Germany (julia.ohmes@uksh.de, andreas.seekamp@uksh.de, sabine.fuchs@uksh.de)
b Protein Chemistry and Enzyme Technology Section, DTU Bioengineering, Department of Biotechnology and Biomedicine, Technical University of Denmark, Building 221, 2800 Kongens Lyngby, Denmark (mdami@dtu.dk, thuthi@dtu.dk, vyha@dtu.dk, asmei@dtu.dk)
c Nha Trang Institute of Technology Research and Application, Vietnam Academy of Science and Technology, 02 Hung Vuong Street, Nhatrang 650000, Vietnam
d Department of Chemistry, Technical University of Denmark, Building 207, 2800 Kongens Lyngby, Denmark (smei@kemi.dtu.dk)
e Orthopedic Research Laboratory, Department of Orthopedic Surgery and Traumatology, Odense University Hospital & Department of Clinical Research, University of Southern Denmark, 5000 Odense C, Denmark (manie15@student.sdu.dk, Ming.Ding@rsyd.dk)

* Correspondence: sabine.fuchs@uksh.de; Tel.: +49-431-500-2-561

Graphical abstract

Hydrolysis of Fucoidan by Fucoidanase Changes Bioactivity in Angiogenic and Inflammatory Processes Relevant for Bone Regeneration

Conclusion

Fucoidan fractions obtained by enzymatic hydrolysis lose their anti-angiogenic properties that have been observed for the native fucoidan HMW F3. In contrast to MMW, LMW does not cause an inflammatory response in endothelial cells. Targeted enzymatic cleavage enables to create different fucoidan molecules which can be screened for desired bioactivities in biological assays. Once the fucoidan of interest is found, the technique can be used to produce the exact same molecule without chemical variations, thereby achieving a consistent bioactivity for future research or applications.

Abbreviations

IEX ion-exchange chromatography, MSC mesenchymal stem cell, IL-6 interleukin-6, ICAM-1 intercellular adhesion molecule 1, VCAM-1 vascular cell adhesion molecule, VEGF vascular endothelial growth factor, ANG-1 angiopoietin 1, ANG-2 angiopoietin 2, OEC outgrowth endothelial cell, HMW high molecular weight, MMW medium molecular weight, LMW low molecular weight
Abstract

Fucoidans are polysaccharides from brown macroalgae, showing multiple bioactivities important for bone regeneration and bone health. However, the use of fucoidans in medical applications remains sparse due to the heterogeneity in their chemical properties and unclear structure-function relationships. Innovations in extraction techniques and post processing steps are needed to produce homogeneous fucoidan molecules with tailorable bioactivities. Here, we applied enzyme-assisted extraction coupled with enzymatic hydrolysis by FhI fucoidanase to generate low (LMW) and medium molecular weight (MMW) fucoidans from Fucus evanescens. In contrast to the anti-angiogenic properties of the high molecular weight fucoidan, LMW and MMW no longer suppressed the production of pro-angiogenic molecules by bone stem cells, nor impaired the formation of prevascular structures in vitro. In contrast to LMW, a pro-inflammatory response of OEC was observed after treatment with high concentrations of MMW. Thus, fucoidanase hydrolysis could be a useful tool to tailor the bioactivity of fucoidans.

Key words

fucoidan, fucoidanase, enzyme-assisted extraction, angiogenesis, inflammation, bone regeneration

1. Introduction

Fucoidans, sulfated polysaccharides from the cell wall of brown macroalgae, are known to influence molecular mechanisms that play a crucial role during bone healing such as inflammation (Park et al., 2011), angiogenesis (Oliveira et al., 2019; F. Wang et al., 2021) and osteogenesis (B. S. Kim, Kang, Park, & Lee, 2015; Pereira et al., 2014). However, the bioactivity of fucoidans relies on the chemical structure which varies depending on algae species, season of harvesting and extraction method (Ale & Meyer, 2013). The large heterogeneity of fucoidans used in bioactivity studies makes results difficult to compare and hence, the relationship between chemical structure and bioactivity of fucoidan still remains unclear. A novel enzyme-assisted fucoidan isolation technique yields reproducible fucoidan extracts with a low batch-to-batch variation and conserves the original structure compared to conventional chemical fucoidan extraction methods. For this technique, the algal cell wall components are digested by cellulases and alginate lyases to release fucoidan. In a subsequent step, the released crude fucoidan is purified into three fractions using ion-exchange chromatography (IEX) (Nguyen et al., 2020). Next to chemical features such as sulfation degree and monosaccharide content, it is known that the bioactivity of fucoidan is also affected by its molecular weight (Gupta et al., 2020; Y. Wang et al., 2019). Fucoidanases, enzymes mainly found in marine bacteria, depolymerize fucoidans at specific sites by highly specific glycosyl hydrolase activity, resulting in chemically defined fucoidan fractions. Fucoidanase digestion has not only the advantage to create fucoidans with defined molecular weights, but also assures consistency of the chemical structure in repeated extractions (Kusaykin, Silchenko, Zakharenko, & Zvyagintseva, 2016).

Bone regeneration is a very complex process consisting of various coordinated molecular biological events involving a wide range of cells like mesenchymal stem cells (MSC), endothelial cells and immune cells. Inflammation, angiogenesis and bone formation are highly interconnected processes and need to occur in perfect balance to ensure successful bone regeneration and bone health maintenance. Angiogenesis, the formation of new blood vessels from already existing ones, is an indispensable process for effective bone regeneration as they transport oxygen, nutrients and minerals.
to the injured site (Stegen, van Gastel, & Carmeliet, 2015). During bone fracture, blood vessels are disrupted, leading to the formation of a hematoma, creating a hypoxic and acidic environment. An acute inflammatory response is triggered to clear the tissue from dead cells, debris and pathogens (Maruyama et al., 2020). Locally stimulated immune cells such as natural killer cells or polymorphonuclear leukocytes release pro-inflammatory cytokines like interleukin-6 (IL-6) which induce the expression of adhesion molecules including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on the surface of endothelial cells (Pober & Sessa, 2007). Leukocytes, attracted through chemokines, bind to these adhesion molecules and transmigrate into the injured bone tissue (Regal-McDonald & Patel, 2020). Synchronized with the initiation of inflammatory events, the hematoma environment also triggers MSC to produce and to release pro-angiogenic mediators such as vascular endothelial growth factor (VEGF) (Folkman & D’Amore, 1996; Grosso et al., 2017). Secreted VEGF triggers endothelial cells to switch from a stable quiescent into an activated state which is characterized by an increase of migration and proliferation. Angiopoietin-2 (ANG-2) is released from activated endothelial cells and acts together with VEGF as an antagonist of angiopoietin-1 (ANG-1) which fosters tight cell-cell adhesion and a low inflammatory state during quiescence (Augustin, Young Koh, Thurston, & Alitalo, 2009; Suri et al., 1996).

In previous publications from our group, mainly high molecular weight fucoidans isolated by different techniques from different algae species were investigated. All tested fucoidan extracts exhibited negative effects on angiogenic processes using the same cell model systems as in the current study (Ohmes et al., 2020; Fanlu Wang et al., 2017; F. Wang et al., 2021). In the study from Ohmes et al., high molecular weight (HMW) fucoidan was isolated from F. evanescentis using the enzyme-assisted extraction technique mentioned above and the bioactivity of the three eluted fractions gained by IEX was compared. The purest fraction (HMW F3, 400-500 kDa) had the highest fucose content and sulfation degree and was proven to impair angiogenic and osteogenic processes, thus potentially interfering with bone repair. Additional enzymatic processing steps could be an option to tailor the bioactivities of fucoidans for specific applications, such as bone regeneration. For the present study, the purest most bioactive fucoidan fraction HMW F3 (400-500 kDa) was further enzymatically processed using a newly discovered fucoidanase obtaining medium molecular weight fucoidans (MMW, peaks at 10 and 200 kDa) and low molecular weight (LMW, 2 kDa) oligosaccharides (Vuillemin et al., 2020). The endo-α-α-(1,4)-fucoidanase Fhf1 from the marine bacterium Formosa halotolisis cleaves α-(1,4)-glycosidic bonds in fucoidans with alternating α-(1,3)-/α-(1,4)-linked L-fucopyranosyls sulfated at C2. All fucoidans were chemically characterized including monosaccharide content and sulfation degree.

The present study compares the bioactivity of fucoidan extracts after fucoidanase hydrolysis on angiogenesis and inflammation in outgrowth endothelial cells (OEC) and MSC mono- and co-culture systems, analyzing whether LMW and MMW fucoidans after enzymatic hydrolysis have a different impact on molecular processes than the HMW F3 fucoidan. We observed that treatment with high doses of MMW causes a pro-inflammatory response indicated by increased IL-6 and ICAM-1 levels, as well as a loss in the integrity of the endothelial cell layer. Additionally, we found that the inhibitory effect on angiogenesis-related mediators is lost after enzymatic hydrolysis of HMW F3 and that the development of angiogenic tube-like structures is no longer impaired by MMW and LMW.
2. Hypotheses

Enzyme-assisted extraction together with defined fucoidanase cleavage represents a technique to tailor fucoidan’s bioactivities and to ensure reproducible chemical properties.

3. Materials and Methods

3.1 Ethical Approval

The use of human material was approved by the local ethics committee of the University Medical Center Schleswig-Holstein. Isolation of primary cells from human tissue was performed with the agreement of the donors.

3.2 Fucoidan isolation from Fucus distichus subsp. evanescens

The upper 2/3 part of over 2 years old brown algae Fucus distichus subsp. evanescens was harvested in March 2017 from 1 m water depth at the Kiel Canal, Germany, and kindly provided by Coastal Research & Management GmbH. Preparation of algal material and fucoidan isolation using an enzyme-assisted extraction technique were performed as described in an earlier publication (Nguyen et al., 2020). Briefly, algal material was washed, lyophilized and grinded, followed by an enzymatic treatment using Cellic®CTec2 cellulase and alginate lyase SALy from Sphingomonas sp. After precipitation with CaCl₂ and ethanol, the crude extract (FE_crude) was purified into three fractions (F1, F2, F3) using IEX.

3.3 Enzymatic cleavage of HMW F3 by fucoidanase Fhf1Δ470

After identifying HMW F3 as the purest and most bioactive fraction in angiogenesis- and osteogenesis-related studies (Ohmes et al., 2020), HMW F3 was chosen for further enzymatic degradation. The hydrolysis of HMW F3 was catalyzed by a recombinantly produced, stabilized version of the the newly discovered endo-α-(1,4)-fucoidanase Fhf1 from Formosa haliotis (Fhf1Δ470). The recombinant expression in E. coli and purification of the enzyme is described in an earlier publication (Vuillemin et al., 2020). For HMW F3 degradation, 800 mg fucoidan were incubated for 24 h with 50 mg/l Fhf1 in 10 mM Tris-HCl (pH = 8) at 37°C. After stopping the reaction by heating at 80°C for 10 min, the reaction products were centrifuged at 20°C and 19000 g for 45 min. The MMW products were separated from the LMW reaction products by precipitation with 75 % ethanol and subsequent centrifugation at 4°C and 19000 rpm for 15 min. LMW was concentrated under vacuum and lyophilized. Absence of bacterial endotoxins in the fucoidan extracts was confirmed by quantitative endotoxin assessment using the Endolisa kit (Hyglos GmbH, Bernfried am Starnberger See, Germany).

3.4 Chemical analysis of MMW and LMW fucoidans

The analysis of monosaccharide composition, sulfate content and molecular weight were described in a previous publication (Nguyen et al., 2020). In brief, high-performance anion-exchange chromatography with pulsed amperometric detection (HPEAC-PAD) was used to determine the sugar composition. The BaCl₂ method was applied to quantify the sulfate content (Dodgson & Price, 1962). The molecular weights of the tested extracts were estimated using high-performance size exclusion chromatography coupled with a refractive index detector. Pullulan was used as a standard.
3.5 $^1$H-$^{13}$C NMR of MMW and LMW fucoidan

NMR spectroscopy was employed in order to gain insight into the chemical structure of the fucoidan fractions. LMW (7.5 mg) and MMW (9.3 mg) were dissolved under agitation in 500 µl $^2$H$_2$O and NMR spectra were acquired at 50°C on an 800 MHz Bruker Avance III instrument equipped with an 18.7 T Oxford magnet and a TCI cryoprobe (5 mm). A suite of 2D NMR experiments was acquired with standard Bruker pulse sequences. Spectra including $^1$H NMR spectra (of 16384 complex data points sampling the FID for 1.7 seconds), $^1$H-$^1$H TOCSY (2048 × 256 complex data points sampling the FID for 128 ms and 16 ms in the direct and indirect dimension, respectively), $^1$H-$^1$H COSY (2048 × 256 complex data points sampling the FID for 128 ms and 16 ms in the direct and indirect dimension, respectively), $^1$H-$^{13}$C HMBC (2048 × 128 complex data points sampling the FID for 256 ms and 6.3 ms, in the direct and indirect dimension, respectively) and $^1$H-$^{13}$C HSQC (2048 × 512 complex data points sampling the FID for 160 ms and 21.2 ms in the direct and indirect dimension, respectively) were acquired. All NMR spectra were processed with ample zero filling in all dimensions and baseline correction using Bruker Topspin 3.5 pl7 software. The spectra were analyzed using the same software.

3.6 Primary cell cultivation and fucoidan treatment

3.6.1 Isolation and cultivation of human outgrowth endothelial cells

OEC were isolated from human peripheral blood as described in previous publications (Fuchs, Hermanns, & Kirkpatrick, 2006). After isolation, OEC were cultured in fibronectin-coated flasks or plates using endothelial growth medium 2, containing endothelial basal medium, associated supplements (Promocell, Heidelberg, Germany), 5 % FBS (Sigma-Aldrich, Steinheim, Germany) and 1 % Penicillin/Streptomycin (Gibco, Grand Island, NY, USA). OEC were sub-cultured every two to three days after reaching confluence. The medium was exchanged every second or third day.

3.6.2 Isolation and cultivation of human mesenchymal stem cells

MSC were isolated from cancellous bone as described in previous publications (Kolbe et al., 2011). After isolation, MSC were cultured in collagen type I-coated flasks using DMEM/Ham’s F-12 (PAN Biotech, Aidenbach, Germany) containing 20 % FBS and 1 % Penicillin/Streptomycin. After the first sub-culture, the FBS content was reduced to 10 %. After the second sub-culture, MSC were differentiated into osteoblast-like cells using osteogenic differentiation medium (ODM) containing DMEM/Ham’s F-12 supplemented with 10 % FBS, 1 % Penicillin/Streptomycin, 50 µM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich) and 0.1 µM dexamethasone (Sigma-Aldrich). The medium was exchanged every second or third day.

3.6.3 Fucoidan treatment of MSC/OEC mono-culture and co-culture

For the mono-culture experiments, OEC or MSC were seeded at a density of 40,000 cells/cm$^2$ in fibronectin-coated 24-well plates or 8 well µ-slides (Ibidi, Gräfelfing, Germany) for cell/supernatant harvest or immunocytochemistry, respectively. For the co-culture experiments, MSC were seeded in fibronectin-coated plates or slides with the same cell density. After 24 h, OEC were seeded with the same cell density in EGM-2 on top of the MSC. On the next day, the culture medium was replaced by culture medium containing 10 or 100 µg/ml fucoidan. After three days, the old media were replaced by fresh media containing the same fucoidan concentrations. Control cells were cultured in the respective media without fucoidan. After seven days of treatment, cells/supernatant was harvested or
cells were fixed for immunocytochemistry. Passage numbers were 4-9 and 4-5 for OEC and MSC, respectively.

3.7 Quantification of gene expression by quantitative real-time PCR

Cells were seeded in 24-well plates and treated with fucoidan as described in section 3.6.3. After seven days of treatment, the cells were incubated with 100 µl/well RNA Lysis Buffer T for 10 min at 37°C. The cell lysate was collected and RNA was isolated using thepeqGOLD Total RNA kit (VWR, Leuven, Belgium) according to the manufacturer’s protocol. DNase I (VWR) treatment for DNA digestion was included into the isolation procedure. 1 µg RNA was transcribed into cDNA using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Vilnius, Lithuania) following the manufacturer’s instructions. The cDNA was diluted in a ratio of 1:5 in nuclease-free water before it was used for qPCR. For determining the gene expression of VEGF, ANG-1, IL-6, ICAM-1, VCAM-1, NFκB and VE-cadherin, 3.2 µl cDNA was mixed with 10 µl SYBR™ Select Master Mix (Applied Biosystems), 2 µl QuantiTect Primer Assays (Qiagen, Hilden, Germany, for more information see Table 1) and 4.8 µl nuclease-free water. RPL13A was used as the housekeeping gene. qPCR was ran with a two-step program (50°C 2 min, 95°C 2 min, 40 cycles 95°C 15 s and 60°C 60 s). The relative gene expression was calculated using the ΔΔcₜ method. All values were plotted relative to the control. The plots show the mean values ± sd for three individual experiments using three different OEC and MSC donors.

Table 1. QuantiTect Primer Assays for qPCR used in the current study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>QuantiTect Primer Assay</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular Endothelial Growth Factor A (VEGF)</td>
<td>Hs_VEGFA_2_SG</td>
<td>QT01036861</td>
</tr>
<tr>
<td>Angiopoietin 1 (ANG-1)</td>
<td>Hs_ANGPT1_1_SG</td>
<td>QT00046865</td>
</tr>
<tr>
<td>Interleukin-6 (IL-6)</td>
<td>Hs_IL6_1_SG</td>
<td>QT00083720</td>
</tr>
<tr>
<td>Intercellular Adhesion Molecule 1 (ICAM-1)</td>
<td>Hs_ICAM1_1_SG</td>
<td>QT00074900</td>
</tr>
<tr>
<td>Vascular Cell Adhesion Protein 1 (VCAM-1)</td>
<td>Hs_VCAM1_1_SG</td>
<td>QT00018347</td>
</tr>
<tr>
<td>Nuclear Factor NF-kappa-B p105 subunit (NFκB)</td>
<td>Hs_NFKB1_1_SG</td>
<td>QT00063791</td>
</tr>
<tr>
<td>Vascular Endothelial Cadherin (VE-cad)</td>
<td>Hs_CDH5_1_SG</td>
<td>QT00013244</td>
</tr>
<tr>
<td>60S Ribosomal Protein L13a (RPL13A)</td>
<td>Hs_RPL13A_1_SG</td>
<td>QT00089915</td>
</tr>
</tbody>
</table>

3.8 Quantification of protein levels by ELISA

Cells were seeded in 24-well plates and treated with fucoidan as described in section 3.6.3. After seven days of treatment, the supernatant was collected. VEGF, ANG-1, IL-6 and ICAM-1 protein levels were quantified from the supernatant using DuoSet® ELISA Development Systems (R&D Systems, Minneapolis, USA) following the manufacturer’s instructions. All values were plotted relative to the control. The plots show the mean values ± sd for three individual experiments using three different OEC and MSC donors.

3.9 Immunocytochemistry of OEC/MSC mono-cultures

Cells were seeded in 8 well µ-slides and treated with fucoidan as described in section 3.6.3. For VE-cadherin staining, the cells were fixed with 4 % paraformaldehyde for 15 min after seven days of treatment. After fixation, cells were permeabilized with 0.5 % Triton™ X-100 (Sigma-Aldrich) for 15 min, followed by blocking of unspecific binding sites using 1 % BSA for 30 min. Human VE-cadherin primary antibody (AF938, R&D) was diluted to 4 µg/ml in 1 % BSA and incubated for 1 h at
room temperature. 2 µg/ml of secondary antibody (AlexaFluor488/anti-goat (A11055, Invitrogen, Waltham, Massachusetts, USA)) was applied for 1 h after 3x 5 min washing with PBS. Nuclei were stained for 15 min with 2 µg/ml Hoechst 33258 (Sigma-Aldrich). After washing 3x for 5 min, the samples were mounted using Fluoromount™ (Sigma-Aldrich) and imaged with the Evos FL Auto 2 fluorescence microscope (Thermo Fisher Scientific, Massachusetts, USA).

3.10 Image analysis

3.10.1 Quantification of angiogenic tube-like structures

MSC and OEC were seeded in 8 well µ-slides and treated with fucoidan as described in section 3.6.3. Seven days after treatment, cells were fixed and stained for VE-cadherin as described in section 3.9. Three stitched pictures consisting of 9 frames were taken for each sample. A shading correction was performed for the stitched images using the BASiC plug-in in FIJI (Peng et al., 2017; Schindelin et al., 2012). Area and length of the angiogenic skeleton were quantified semi-automatically using the software ImageJ Version 1.42 (Schneider, Rasband, & Eliceiri, 2012) as described in a previous publication (Fuchs et al., 2009). The plots show the mean values ± sd for three individual experiments using three different OEC and MSC donors.

3.10.2 Quantification of the integrity of the endothelial cell layer

MSC and OEC were seeded in 8 well µ-slides and treated with fucoidan as described in section 3.6.3. Seven days after treatment, cells were fixed and stained for VE-cadherin as described in section 3.9. Three stitched microscopy pictures consisting of 9 frames were taken for each sample and corrected as stated in section 3.10.1. The integrity of the endothelial cell layer was expressed by quantifying the area which was not covered by OEC using FIJI (Schindelin et al., 2012). Therefore, fluorescent images were converted into binary images and black areas (areas without OEC) were measured. The plots show the mean values ± sd for three individual experiments using three different OEC and MSC donors.

3.11 Statistical analysis

ANOVA with post-hoc Dunnett test was used to calculate the statistical significances in GraphPad Prism 7.03. Values were considered as significant when p < 0.05.
4. Results

4.1 Chemical characterization of the fucoidan extracts

Brown macroalgae material from *Fucus distichus* subsp. *evanescens* was digested by cellulases and alginate lyases to release crude fucoidan (FE_crude). FE_crude was purified by ion-exchange chromatography obtaining three eluted HMW fractions (F1, F2, F3). A study comparing the chemical properties and bioactivities of the crude extract and the three fractions in the context of angiogenesis and osteogenesis was described earlier (Ohmes et al., 2020). The purest fraction HMW F3, containing the highest fucose content, affected angiogenesis- and osteogenesis-related processes most. HMW F3 treatment reduced the gene expressions and protein levels of angiogenic mediators, such as VEGF and ANG-1 in MSC, and impaired the formation of angiogenic structures in MSC-OEC co-culture. Therefore, HMW F3 was chosen as the substrate for hydrolysis, catalyzed by the recently discovered endo-fucoidanase Fhf1. Fhf1 cleaves α-(1,4)-glycosidic bonds between non-acetylated and C2-monosulfated units in fucoidans with alternating α-(1,3)/α-(1,4)-linked L-fucopyranosyls yielding LMW oligosaccharides and a MMW fraction (Vuillemin et al., 2020). The fucoidan extraction and enzymatic processing are schematically illustrated in Figure 1.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Overview of the enzyme-assisted fucoidan extraction and fucoidanase processing. Brown alga *Fucus distichus* subsp. *evanescens* (FE) was treated with cellulases and alginate lyases to extract crude fucoidan (FE_crude). The crude extract was purified into 3 fractions (F1, F2, and F3) using ion-exchange chromatography (IEX). The purest most bioactive fraction was HMW F3 (Ohmes et al., 2020), which was hydrolyzed by the endo-fucoidanase Fhf1Δ470 from *Formosa haliotis*. The medium molecular weight reaction product (MMW) was precipitated with ethanol. The remaining low molecular weight oligosaccharides (LMW) were concentrated and lyophilized.

MMW and LMW were analyzed for monosaccharide composition and sulfate content. The results for MMW and LMW, including sugar composition and sulfate content for HMW F3 are listed in supplementary Table A1. All tested fucoidan extracts were mainly composed of fucose (> 87 %). The compositional analysis showed that the LMW fraction was devoid of galactose and mannose. HMW F3 and MMW contained 8.5 % and 7.9 % galactose, respectively, while LMW only contained 1.2 % galactose. Rhamnose, glucose and mannose represented only <1 % of the monosaccharides. All tested extracts contained between 1.2 and 2.7 % xylose. The alginate content (mannuronic acid, guluronic acid) was <1 % in all tested extracts. The overall sulfate content was lower in the LMW (~34 %) compared to the MMW fucoidan (~50 %). These results are consistent with the specificity of Fhf1 fucoidanase, which cleaves mono-sulfated fucosyl residues, leaving the disulfated residues in the MMW fraction, thus resulting in a higher sulfate content in the MMW compared to the LMW fraction.

To estimate the molecular weight of the tested extracts, high-performance size exclusion chromatography was performed. The chromatogram shown in supplementary Figure A1 indicates that extract HMW F3 mainly contains fucoidans with a molecular mass between 400 and 500 kDa and...
therefore belongs to the group of high molecular weight fucoidans. The hydrolysis reaction product MMW is a medium molecular weight fucoidan with two peaks around 10 kDa and 209 kDa. The purified hydrolysis products are oligosaccharides with a size of approximately 2 kDa, belonging to the group of low molecular weight fucoidans.

To determine the chemical structures of the fucoidan products after fucoidanase hydrolysis, NMR spectroscopy was applied. As shown in Figure 2 A), the MMW fraction was found to consist of a repetitive and regular polysaccharide that is largely devoid of acetylation and is disulfated in →3)-α-L-Fucp2,4-di-S-(1→ units. The LMW fucoidan proved to be an oligosaccharide fraction predominantly consisting of tetrasaccharides, sulfated only at C2 of each fucose unit and C3-acetylated at the →4)-α-L-Fucp2S,3OAc-(1→ residue adjacent to the reducing end (as shown in Figure 2 B)). In order to gain additional insights into the architecture of the fucoidan fractions, NMR spectra were further evaluated (see Supplementary Figure A3). The evaluation showed that MMW contains signals that are consistent with the presence of →3,4)-α-L-Fucp(2SO3)-(1→3)- branch points (Clément et al., 2010) at low density of approximately 2.4 % on monomer basis (approximately one in 40 units). In contrast, branching could not be identified in the LMW fraction. The sulfate content deriving from these structures (MMW has a higher sulfate content than LMW) is consistent with the titrimetric BaCl2 sulfate determination. The corresponding NMR spectra for MMW and LMW can be found in supplementary Figure A2.

Figure 2. The chemical structures of fucoidanase hydrolysis products A) MMW and B) LMW based on NMR analysis. (NMR spectra are displayed in Supplementary Figure A2.)

4.2 Effect of fucoidan hydrolysis products on angiogenic mediators in MSC mono-cultures

While ANG-1 is released from MSC to stabilize existing blood vessels (Augustin et al., 2009), VEGF initiates the formation of new blood vessels and is also a regulator for bone cell differentiation (Grosso et al., 2017) and immune cell recruitment (Barleon et al., 1996). To study the effect of molecular weight, respectively the bioactivity changes of MMW and LMW compared to HMW F3 on angiogenic mediators, MSC mono-cultures were treated with the fucoidan extracts for seven days. Subsequently, gene expression and protein level of angiogenic markers VEGF and ANG-1 were quantified. The effect of HMW F3 on the mentioned mediators was published earlier in a different context (Ohmes et al., 2020). For an easier comparison, gene expression and protein level after 100 µg/ml HMW F3 treatment and corresponding controls were included into the result plots. As shown in Figure 3 A), treatment with both concentrations of MMW and LMW had no effect on the VEGF gene expression. In contrast, VEGF gene expression was decreased after treatment with HMW F3. The difference in VEGF protein levels after MMW, LMW and HMW F3 treatment was more prominent; VEGF levels in HMW F3-
treated MSC were approximately 8 times lower than in MMW-/LMW-treated MSC. While 100 µg/ml HMW F3 treatment decreased the VEGF protein levels to less than 10 %, only 100 µg/ml MMW reduced the VEGF levels significantly compared to the control. As shown in Figure 3 B), none of the tested fucoidan treatments, including HMW F3, affected gene expression levels of ANG-1. Similar to VEGF protein levels, HMW/LMW treatment had only a small impact on ANG-1 protein levels, whereas HMW F3 decreased ANG-1 levels to around 30 %. The quantification of gene expression and protein levels of VEGF and ANG-1 demonstrates that treatment with LMW and MMW fucoidans has a weaker effect on the mentioned mediators than HMW F3.

Figure 3. The effect of MMW and LMW treatment on angiogenic mediators in MSC mono-culture. MSC were treated for seven days with 10 and 100 µg/ml MMW or LMW. Control cells were cultured in regular growth medium without fucoidan. The gene expression and protein level of A) vascular endothelial growth factor (VEGF) and B) angiopoietin-1 (ANG-1) were determined using qPCR and ELISA, respectively. For a better comparison, all graphs include the gene expression/protein level of VEGF and ANG-1 in MSC mono-culture after seven days of treatment with HMW F3 as published in (Ohmes et al., 2020). The plots show the mean values ± sd for three individual experiments using three different MSC donors. Significances compared to the control cells (*) and to HMW F3-treated cells (+) were calculated using ANOVA with post-hoc Dunnet (*+/ p < 0.05, **+/+ p < 0.01, ***/+++ p < 0.001, ****/++++ p < 0.0001).
4.3 Effect of fucoidan hydrolysis products on inflammatory processes in OEC monocultures

An inflammatory response is one of the first crucial steps to initiate bone repair. A lasting chronic inflammation however interferes with bone health (Fullerton & Gilroy, 2016; Maruyama et al., 2020). Pro-inflammatory cytokines like IL-6 induce endothelial intracellular pathways such as the NFκB pathway which amongst others regulate the expression of adhesion molecules (ICAM-1, VCAM-1). These adhesion molecules serve as anchor points for monocyte transmigration (Regal-McDonald & Patel, 2020).

![Figure 4](image_url)

Figure 4. The effect of MMW and LMW treatment on inflammatory mediators in OEC monoculture. OEC were treated for seven days with 10 and 100 µg/ml MMW or LMW. Control cells were cultured in regular growth medium without fucoidan. A) The gene expression of interleukin-6 (IL-6), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and nuclear factor κB (NFκB) and B) protein level of IL-6 and ICAM-1 were determined using qPCR and ELISA, respectively. For a better comparison, the gene expression of IL-6, ICAM-1 and VCAM-1 and the protein levels of IL-6 and ICAM-1 in OEC monoculture after seven days of treatment with the HMW F3 were included. The plots show the mean values ± sd for three individual experiments using three different OEC donors. Significances compared to the control cells (*) and to HMW F3-treated cells (+) were calculated using ANOVA with post-hoc Dunnet (*/+ p < 0.05, **/++ p < 0.01, ***/+++ p < 0.001, ****/++++ p < 0.0001).
To determine the effect of molecular weight and the bioactivity changes of MMW and LMW compared to HMW F3 on inflammatory mediators, OEC mono-cultures were treated with the fucoidan extracts for seven days. Subsequently, gene expression of inflammation-related mediators IL-6, ICAM-1, VCAM-1 and NFkB was quantified. Additionally, protein levels of IL-6 and ICAM-1 were determined. For a better comparison, gene expression of IL-6, ICAM-1 and VCAM-1, as well as protein levels of IL-6 and ICAM-1 after treatment with HMW F3 were included into the plots. As shown in Figure 4 A), 10 µg/ml MMW and 10 and 100 µg/ml LMW treatment had no impact on the expression of all tested inflammatory mediators. However, 100 µg/ml MMW treatment increased the expression of ICAM-1 and VCAM-1, indicating a pro-inflammatory response to high doses of MMW. Contrarily, IL-6, ICAM-1 and VCAM-1 expressions were downregulated almost completely in OEC after 100 µg/ml HMW F3 treatment. Figure 4 B) shows that treatment with LMW did not influence the protein level of IL-6, nor ICAM-1, while ICAM-1 protein levels were increased after MMW treatment. These results are in accordance with the expression of the corresponding genes. Similar to the gene expression results, IL-6 and ICAM-1 protein levels were highest after treatment with 100 µg/ml MMW, while IL-6 protein levels were lowest after treatment with HMW F3. The experiment shows that treatment with high concentrations of MMW causes a pro-inflammatory response indicated by increased gene expression and protein levels of the mentioned inflammatory mediators.

4.4 Effect of fucoidan hydrolysis products on the integrity of the endothelial cell barrier in OEC-MSC co-cultures

During an inflammatory response, endothelial cells loosen their cell junctions to enable the extravasation of immune cells from the blood stream into the affected tissue. Loosening of endothelial contacts and the reorganization of the endothelial cell layer is also crucial during early angiogenesis, indicating the similarities between these molecular processes. To study if the observed inflammation triggered by high doses of MMW fucoidan treatment as stated above also affects the integrity of the endothelial cell layer, OEC were co-cultured with MSC and treated with the hydrolyzed fucoidan extracts for seven days. After seven days, the cells were fixed and stained for VE-cadherin. As depicted in Figure 5 A), control OEC form a tight stable layer that covers the majority of the area. The MMW-treated co-cultures however, exhibited larger areas without OEC. The analysis of the area without OEC presented in Figure 5 B) proves that co-cultures treated with 100 µg/ml MMW contain 4 times larger areas without OEC than the control cells. Co-cultures treated with 10 µg/ml MMW and 100 µg/ml LMW show least gaps between OEC, indicating the maintenance of a stable endothelial cell layer after treatment. In accordance with the described results, the gene expression of VE-cadherin was decreased in co-cultures treated with 100 µg/ml MMW, while VE-cadherin expression in the other samples remained at control level (Figure 5 C)). The analysis of the OEC layer integrity indicates that OEC treated with high doses of MMW react not only with an inflammatory response, but also loosen VE-cadherin-associated cellular junctions.

4.5 Effect of fucoidan hydrolysis products on the development of angiogenic structures in OEC-MSC co-cultures

After the acute immune response in early phases of bone repair, the cross-talk between MSC and endothelial cells should result in the formation of stable blood vessels in later stages of the regenerative process. Sufficient vascularization is a prerequisite for successful bone healing (Stegen et al., 2015). To illustrate the effect of molecular weight and the bioactivity changes of MMW and LMW compared to HMW F3 on the formation of angiogenic tube-like structures, OEC-MSC co-cultures were treated with the fucoidan extracts for seven days and subsequently fixed and stained for VE-cadherin.
Figure 6 A) shows exemplary microscopy pictures of VE-cadherin-stained MSC-OEC co-cultures with emerged angiogenic structures after fucoidan treatment. Treatment with the fucoidan extracts MMW and LMW obtained after hydrolysis had no negative effect on the establishment of tube-like structures. In contrast, 100 µg/ml HMW F3 treatment suppressed the development of angiogenic structures almost completely. For some donor cells, the number of angiogenic structures was even increased after treatment with MMW or LMW. Figure 6 B), showing the quantification of area and length of the angiogenic structures, confirms the observation from the microscopy pictures. The length of angiogenic structures was decreased after treatment with 100 µg/ml HMW F3, while treatment with MMW and LMW did not change area and length of the established structures. The experiment shows that treatment with MMW and LMW has no inhibitory effect on the development of angiogenic structures in MSC-OEC co-cultures. This observation indicates that enzymatic processing holds the potential to tailor the bioactivity of fucoidan extracts in regard to effects on angiogenic processes.

Figure 5. The effect of MMW and LMW treatment on the integrity of the endothelial cell layer in OEC-MSC co-culture. MSC and OEC were co-cultured and treated with 10 and 100 µg/ml MMW or LMW for seven days. Control cells were cultured in regular growth medium without fucoidan. A) The co-cultures were stained for endothelial marker VE-cadherin (green) after seven days of treatment and imaged with a fluorescence microscope. Exemplary images for control, MMW- and LMW-treated cells are displayed (scale bars = 300 µm). B) OEC-free areas were quantified for 3 stitched images consisting of 9 frames for each donor. C) The gene expression of VE-cadherin was determined after seven days of treatment using qPCR. The plots show the mean values ± sd for three individual experiments using three different OEC and MSC donors. Significances compared to the control cells were calculated using ANOVA with post-hoc Dunnet (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).
Figure 6. The effect of MMW and LMW treatment on the formation of angiogenic tube-like structures in OEC-MSC co-culture. MSC and OEC were co-cultured and treated with 10 and 100 µg/ml MMW or LMW for seven days. Control cells were cultured in regular growth medium without fucoidan. A) The co-cultures were stained for endothelial marker VE-cadherin after seven days of treatment and imaged with a fluorescence microscope. The images are inverted and displayed in grey values (scale bars = 300 µm). B) The area and length of the formed angiogenic structures were quantified for 27 frames of each cell donor. For a better comparison, the figure includes the results of co-cultures treated with 100 µg/ml HMW F3. The plots show the mean values ± sd for three individual experiments using three different MSC and OEC donors. Significances compared to the control cells (*) and to HMW F3-treated cells were calculated using ANOVA with post-hoc Dunnet (p < 0.05).
5. Discussion

This work investigates whether guided enzymatic hydrolysis by the newly discovered and highly specific endo-fucoidanase Fhf1 can change bioactivity of fucoidan in angiogenesis and inflammation, both processes relevant for successful bone regeneration and maintenance of bone health.

HMW F3 was isolated from *F. evanesens* using an enzyme-assisted extraction technique, followed by IEX purification. The third eluted fraction HMW F3 was cleaved at α-(1,4)-glycosidic bonds between non-acetylated and C2-monosulfated units using the endo-fucoidanase Fhf1 obtaining LMW fucoidan oligosaccharides and a MMW fucoidan fraction. Higher yield, less energy consumption and preservation of the native structure are only few advantages that make enzyme-assisted fucoidan extraction techniques stand out against conventional isolation methods which mostly use treatment with hot water, acidic or salty solvents for several hours at high temperatures (Dobrinčić et al., 2020). Enzymatic extractions of fucoidans have been shown previously where Alboofetileh and colleagues used alcalase to extract fucoidan from *Nizamuddinia zanardinnii*, or Oh and colleagues applied celluclast to isolate fucoidan from *Undaria pinnatifida* sporophylls (Alboofetileh, Rezaei, Tabarsa, & You, 2019; Oh et al., 2020). In the presented work, cellulase and alginate lyase were applied to release fucoidan from the cell wall.

An even newer concept to create fucoidans with specific chemical structures is the use of characterized fucoidanases that specifically cleave polysaccharides into smaller fragments. Different fucoidanases have been discovered in marine organisms, but only few of them are functionally characterized (Kusaykin et al., 2016). For example, fucoidanase found in different *Pseudoalteromonas citrea* strains cleaves α-(1,3)-glycosidic bonds in fucoidans from *F. evanesens*. Sichert and colleagues analyzed ‘Lentimonas’ sp. CC4 for fucoidanase expression and found more than 200 putative fucoidanases (Sichert et al., 2020). While the discovery and characterization of fucoidanases keep progressing, the number of studies investigating the bioactivities of the hydrolyzed fucoidan fragments is still very limited.

In the current study, we treated human microvascular endothelial cells (OEC) and human MSC with LMW and MMW fucoidan fractions obtained after fucoidanase Fhf1 hydrolysis. Expression and production of angiogenic and inflammatory mediators were determined, the integrity of the endothelial cell layer was quantified and the formation of angiogenic structures in MSC-OEC co-cultures was analyzed. In our previous study, we showed that HMW F3 negatively affects mediators important for angiogenesis and osteogenesis such as VEGF and angiopoietins, and also impairs the formation of angiogenic structures (Ohmes et al., 2020). As we show in the current study, the MMW and LMW fucoidans obtained by hydrolysis have no longer an inhibiting effect on angiogenesis. While treatment with HMW F3 decreased VEGF and ANG-1 gene expression and protein level in MSC, treatment with MMW and LMW had only a minor effect on these mediators. Treatment with HMW F3 suppressed the formation of tube-like angiogenic structures in the co-culture model almost completely, while MMW and LMW did not impair the formation of these structures.

Recently, a study was published investigating the effect of fucoidanase-extracted fucoidan oligosaccharides from *F. evanesens* in combination with hydroxyapatite on bone regeneration in sheep after insertion into a critical-sized defect. In the study by Nielsen et al., new bone formation was analyzed by micro-CT and immunohistochemistry (Nielsen et al., 2021). However, there are only few studies comparing the impact of chemically characterized fucoidanase-degraded fucoidan fractions
with the native high molecular weight fucoidan. Chen and colleagues found that fucoidans from *Laminaria japonica* increased the tyrosinase and melanogenesis inhibition, as well as DPPH radical scavenging activities after degradation by fucoidanase from *Flavobacterium RC2-3* (Chen, Kou, Wang, & Wang, 2019). Kim and colleagues observed that enzymatically degraded fucoidans from *U. pinnatifida* had 3-4 times reduced anticoagulating activities and did not influence the prothrombin time compared to the native fucoidan (W. J. Kim et al., 2010). Silchenko and colleagues used fucoidanase FFA1 from *Formosa alga* to degrade fucoidan from *Sargassum horneri* and showed that oligosaccharides were not able to suppress the formation of cancer cell colonies compared to the native fucoidan which inhibited colony formation by 50 % (Silchenko et al., 2017). In conjunction with these previous studies, our work suggests that fucoidanase hydrolysis changes the bioactivity of the resulting fucoidan fractions compared to the native counterpart. While HMW F3 reduced the expressions and protein levels of angiogenic mediators and impaired the formation of angiogenic structures, this effect was not observed for hydrolysis products MMW and LMW.

The most obvious difference between HMW F3 and the fucoidan fractions after hydrolysis is the molecular weight. Many studies demonstrate that molecular weight has a significant impact on the studied bioactivity (Álvarez-Viñas, Flórez-Fernández, González-Muñoz, & Domínguez, 2019; Gupta et al., 2020; Yang et al., 2008), however it remains difficult to assign specific bioactivities to defined molecular weights. In accordance with our results, several publications report that bioactivities are lost or reduced if the molecular weight is too small. Fucoidan oligosaccharides showed reduced anticoagulating activities (W. J. Kim et al., 2013), didn’t suppress cancer cell colony formation (Silchenko et al., 2017), and had a decreased anticoagulating affinity (W. Jin et al., 2020). By visualizing the interaction of the studied fucoidans before and after hydrolysis with our cell models, we found that the interaction of LMW and MMW fucoidans with MSC and OEC was much weaker than for HMW F3 (see Supplementary Figure A). It is known that heparan sulfate, a part of heparin sulfate proteoglycans located on the cellular surface, plays a crucial role during angiogenic events by modulating VEGF and FGF receptor affinity (Gallagher, 2015). Due to their similarity to heparan sulfate, fucoidans bound to the cellular surface could be able to alter the interaction of growth factors with its receptors and therefore affect downstream signaling pathways.

Chronic inflammation is a major problem in bone regeneration and health, which can be caused by diseases, bacterial infections or wear particles after total joint replacement (Croes, van der Wal, & Vogely, 2019; Jiao, Xiao, & Graves, 2015; Lin et al., 2014). For bone repair, applied bioactive compounds should not cause a sustained inflammation. Therefore, we investigated the effect of the fucoidan fractions MMW and LMW obtained after hydrolysis on inflammation. In fact, we found that higher doses of MMW triggered an inflammatory response in OEC indicated by increased expression and production of IL-6 and ICAM-1. Additionally, we observed the loosening of VE-cadherin-associated cell junctions, underlining a pro-inflammatory response after treatment with 100 µg/ml MMW. While numerous studies prove the anti-inflammatory potential of fucoidan extracts (Jayawardena et al., 2020; Ni et al., 2020; Park et al., 2011), only few studies exist indicating a pro-inflammatory effect caused by fucoidan treatment. Jin and colleagues studied the adjuvant function of fucoidan from *Fucus vesiculosus* and found that fucoidan induced the production of pro-inflammatory cytokines in dendritic cells. Fucoidan in combination with ovalbumin antigen enhanced the antibody production, upregulated MHC class I and II and increased T cell proliferation *in vivo* (J. O. Jin et al., 2014). The same authors published in another study that fucoidan from *U. pinnatifida* increased the production of IL-6, IL-8 and TNF-α in neutrophils (J. O. Jin & Yu, 2015).
Further, potential bacterial impurities in fucoidan extracts like endotoxins can cause inflammatory reactions. However, the quantification of endotoxins resulted in very low levels in MMW and LMW (see Supplementary Table A2), excluding bacterial impurities originating from the extraction process or the production of recombinant fucoidanase Fhf1. If endotoxins due to extraction or hydrolysis can be excluded, the different effect on inflammation of MMW and LMW must likely be found in the chemical structure. Next to molecular weight, the extracts differ in sulfate content and pattern, acetylation and charge. Due to a slightly increased negative charge and molecular weight, the MMW fraction and its conformation have stronger characteristics of a polyanion than the LMW oligosaccharides. MMW is disulfated at C2 and C4 and contains therefore a higher sulfate content than LMW which is only sulfated at C2. Higher sulfate content is often associated with changes in bioactivity. Hsiao and colleagues suggest that oversulfated fucoidan facilitates apoptosis in lung cancer cells (Hsiao et al., 2021). We showed in a previous study that expression of anti-angiogenic and anti-osteogenic mediators was enhanced by fucoidans with a higher degree of sulfation (Ohmes et al., 2020). The sulfate content determines the charge of the fucoidan molecule which in turn can be responsible for protein affinity. Koyanagi and colleagues showed that oversulfated fucoidan was able to form stable complexes with VEGF-165 preventing the binding to its receptor (Koyanagi, Tanigawa, Nakagawa, Soeda, & Shimeno, 2003). Apart from degree of sulfation, also the sulfation pattern could have an influence on the bioactivity. The monosulfation at C2 in the LMW fraction might create an almost linear tetrameric structure, thereby having a different bioactivity than MMW.

While a lot of literature exists claiming the importance of sulfates in regard to bioactivity, the impact of acetylation is less studied. NMR analysis revealed that LMW oligosaccharides studied in the presented work were acetylated at C3, while the MMW fucoidan fraction did not contain acetylations at any significant level. Wang and colleagues produced an acetylated fucoidan derivate and found that the antioxidant effect of the derivate was stronger compared to the native fucoidan (J. Wang et al., 2009). On the other hand, Lapikova and colleagues observed that native fucoidan, as well as the deacetylated derivate were both able to inhibit thrombin and factor Xa to the same extent, concluding that acetylation was not a determining factor for the observed bioactivity (Lapikova et al., 2008). The lack of studies reporting on a pro-inflammatory effect after fucoidan treatment makes it difficult at this stage to identify chemical characteristics which are responsible for the observed effects. Based on the results, it seems likely that only the interplay of multiple chemical parameters can explain the different bioactivities observed in the presented study. In addition to molecular weight and sulfate content probably other properties like 3D structure and branching play a role. While fucoidans from F. evanesens were originally considered as linear polymers (Bilan et al., 2002), this assessment was later revised as some →3,4)-α-L-Fucp(1→3)- branch points were identified on desulfated and deacetylated samples (Bilan, Grachev, Shashkov, Nifantiev, & Usov, 2006). NMR analysis presented in this study indeed showed signals in the MMW fraction that indicate the presence of branching at low density. These branch points affect the structure and the local charge density and therefore probably also the bioactivity of this fraction. In contrast, the LMW fraction contains short, unbranched units with a lower charge density than the MMW fraction. The impact of branching on the bioactivity of fucoidans was shown earlier. For example, Clément and colleagues demonstrated that branched fucoidan oligosaccharides from Ascophyllum nodosum had a higher anticomplementary activity than the linear counterparts (Clément et al., 2010).

In conclusion, MMW and LMW fucoidans resulting from enzymatic degradation of HMW F3 from F. evanesens showed altered bioactivities in endothelial cells and bone mesenchymal stem cells. MMW
and LMW lost the anti-angiogenic properties which were reported for HMW F3 before. In contrast to HMW F3, LMW and MMW did not impair the secretion of pro-angiogenic molecules in MSC and did not interfere with the formation of microvessel-like structures in MSC-endothelial cell co-cultures. Further, in contrast to MMW, LMW did not provoke an inflammatory activation in endothelial cells. From all fucoidans which were tested so far in the presented cell model systems, LMW represents the most suitable extract for further adaptations and studies. The presented results highlight the potential of enzymatic processing techniques to select and tailor fucoidans for specific biomedical applications and to elucidate structure-function relationships in the context of bone regeneration.

Declarations of interest: none

Author contributions:
Julia Ohmes: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Original draft
Maria Dalgaard Mikkelsen: Conceptualization, Methodology, Project administration, Supervision, Review & editing
Thuan Thi Nguyen: Data curation, Formal analysis, Investigation, Methodology
Sebastian Meier: Data curation, Formal analysis, Methodology, Review & editing
Mads Suhr Nielsen: Review & editing
Ming Ding: Project administration, Supervision, Review & editing
Andreas Seekamp: Funding acquisition, Project administration, Supervision
Anne S. Meyer: Funding acquisition, Project administration, Supervision, Review & editing
Sabine Fuchs: Conceptualization, Funding acquisition, Project administration, Supervision, Review & editing, Methodology

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References


Figure 1
Figure 2
Figure 3

A) Gene expression and protein level of VEGF

B) Gene expression and protein level of ANG-1
Figure 6