Coagulation Activity of Human Blood Plasma in the Presence of Ultrasound-Processed Fucoidan

Victoria Suprunchuk*
Department of Pharmaceutical Chemistry and Drug Technology, North-Caucasus Federal University, Stavropol, Russia.

ABSTRACT

AIM
To evaluate the effect of low-frequency ultrasound with variable parameters and environments on biological activity of fucoidan within determination of human plasma coagulation values.
METHODS
The study was performed using fucoidan derived from the tangle Fucus vesiculosus. Fucoidan was processed with a high-intensity low-frequency ultrasound in water environment with and without administration of surface-active substances. The effectiveness and change in biological activity of the polysaccharide was evaluated using clotting tests: Prothrombin Test (PT test), partial thromboplastin test (APTT test), and Thrombin Test (TT test).
RESULTS
During this study, it was determined that fucoidan can extend the clotting time of human plasma *in vitro* both before and after ultrasound processing. Higher values were detected for fucoidan processed with the administration of surface-active substances. Following sonochemical processing, decreased fucoidan activity was detected by TT and APTT tests compared to the original polysaccharide. Increased ultrasound intensity leads to retained biopolymer activity in the APTT test. During determination of methods of coagulation process inhibition, the effect of processed fucoidan on the external and internal ways of blood clotting was confirmed. As a result of the study, the most favorable conditions for hydroacoustic processing aimed at maintaining the biological activity of fucoidan were determined.
CONCLUSION
Based on the obtained data, we can suggest that using fucoidan after sonochemical processing in the development of thrombolytic therapy products can be advantageous.
KEYWORDS
Fucoidan, Ultrasound processing, Depolymerization, Anticoagulation activity

Corresponding Author:

Victoria Suprunchuk, Department of Pharmaceutical Chemistry and Drug Technology, North Caucasus Federal University, Stavropol, Russia.
Email: vsuprunchuk@ncfu.ru

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INTRODUCTION

Fucoidan is a highly sulphated heteropolysaccharide with properties similar to those of heparin. This polysaccharide is characterised by a high availability of its source, that is, tangles, with a wide spectrum of biological activity, which makes this study advantageous in terms of its introduction into clinical practice. The polysaccharide is known for its heparin-like effect with expressed anticoagulation properties.[1] However; the molar weight of this polysaccharide is highly variable. Fucoidan can be classified as low-molecular (3 to 8 kDa) and high-molecular (more than 2000 kDa). As a rule, the high molecular type is represented by native fucoidan with large molecules, leading to low permeability of the cell membrane and decreased effectiveness. Some types of activity were detected to increase with decreasing polymerization, which may be associated with increased solubility of the polysaccharide in water.[2] Decreasing the polymerization of polysaccharides is possible using several approaches, including ultrasound processing. Ultrasound processing has been studied for many polysaccharides (such as chitosan,[3] dextran,[4,5] cellulose[6]), showing high effectiveness. Using this method of biopolymer degradation, a polysaccharide water solution can be obtained without the administration of toxic solvents and with a saving effect on the chemical structure of the polysaccharide[7] and a limited molar weight distribution.[8] In addition, ultrasound increases the biological activity of polysaccharides.[9] Thus, it was detected that antioxidant activity increased after ultrasound processing of yellow-tea polysaccharide.[10] At the same time, in general, the relevant structural characteristics of individual polysaccharides are retained, such as acidylation and methylation grade (for pectin) and monosaccharide composition.[11] However, fucoidan ultrasound destruction was considered only for some of its types, such as fucoidan derived from the sea gherkin Isostichopus badionotus,[10] for which polysaccharide anticoagulation activity was not studied. Based on the above, this study aimed to determine how anticoagulation activity of human plasma changes in the presence of ultrasound processed fucoidan. During the study, varying parameters of tangle-derived ultrasound processing of tangle-derived polysaccharide were used to determine the most favorable ones. We also supposed that administration of surface-active substances into the processing environment would increase solubility of the polysaccharide in water and, respectively, the effect of ultrasound, and also decrease the radical effect of sonolysis through stabilization of H_2O_2 created during the ultrasound processing. We used ionogenic (SDS) and non-ionogenic (PEG-400) surface-active substances.

MATERIALS and METHODS

Isolation of Fucoidan from Brown Algae

For ultrasonic treatment, we used a polysaccharide obtained from the brown alga Fucus vesiculosus according to the method presented in work,[12] with some modifications. The subsequent purification was carried out in accordance with the work of A. M. Urvantseva et al.[13]

Ultrasonic Treatment of Fucoidan

The obtained biopolymer was dispersed in deionized water at a concentration of 10 mg / ml using an ultrasonic homogenizer UIP 1000 hd (20 kHz, 250 W / cm^2) (Hielscher Ultrasonics GmbH, Germany) with a maximum intensity of 250 W / cm^2 and a frequency of 20 kHz. Cooling was carried out using an ice bath. During the study, the amplitude was varied with a value of 20 %, 40 %, 80 % of the working cycle, which corresponds to an intensity of 100, 133, 200 W / cm^2, respectively, with a period of 40 min (t) in the absence and introduction of surfactants. The temperature was monitored using a thermal sensor and did not exceed 313 K.

Coagulation Testing of Human Blood Plasma in the Presence Of Fucoidan

All clotting tests were performed using an EMCO coagulometer (APG 4-03-Px). At the end of coagulation, the measured clotting time was expressed in seconds. As a control, 50 µl of human blood plasma containing 10 µl of 0.9 % NaCl was taken. Determination of clotting time (PT test) was performed using 100 µl of thromboplastin-calcium. Determination of activated partial thromboplastin time (APTT test) was performed using 50 µl of the APTT reagent and 50 µl of 0.025 M $CaCl_2$ (at 37 °C for 30 min). Determination of thrombin clotting time (TT test) was performed using 50 µl of thrombin.

Determination of Antimicrobial Activity

The study of antibacterial activity was carried out by the disk diffusion method[14] with some modifications. Briefly, *Escherichia coli* and *Staphylococcus aureus* were cultured at 37 °C after 24 h on meat peptone agar (peptone 5.0, meat extract 3.0, agar 15.0). Before incubation, a sterile disk (5 mm) was loaded with 10 µg / ml of simple fucoidan and dried at room temperature under sterile condition in a Petri dish. Then, the zone of inhibition was measured.

RESULTS AND DISCUSSION

Modification of polysaccharides after ultrasound processing can serve as a basis for changing its

biological characteristics. Thus, the anticoagulation activity of fucoidan is directly related to the molar weight of the polymer. Fucoidan has an effect on internal and external coagulation ways,[15] however, the mechanism of it action depends on the molar weight of the biopolymer. Thus, fucoidan with a low molar weight activates the antithrombin and heparin cofactor II, while fucoidan with a high molar weight interacts directly with thrombin[16] provided that there is a conformation suitable for thrombin fixation.[17]

To determine anticoagulation activity and mechanism of action of ultrasound-processed fucoidan, PT, TT and APTT clotting tests were used. For these tests, the following fucoidan water solutions were used: non-processed (original) (I); processed with ultrasound intensity 100 W / cm^2 (II), 133 W / cm^2 (III), 200 W / cm^2 (IV); processed with ultrasound intensity 133 W / cm^2 but in the presence of SDS (III) and PEG-400 (III) (Table 1).

Sample	Conditions for obtaining (I Вт/см^2; SAS)	PT, c	TT, c	APTT, c
I	-	22,2 ± 1,02	16,49 ± 0,29	51,9 ± 1,71
II	100	28,7 ± 0,98	15,81 ± 0,49	45,7 ± 1,51
III	133	32,8 ± 1,44	15,13 ± 0,53	44,5 ± 1,47
IV	200	33,4 ± 1,49	20,15 ± 0,34	52,8 ± 1,78
III´	133; SDS	50,6 ± 1,73	20,63 ± 0,98	51,1 ± 1,69
III ˝	133; PEG-400	53,2 ± 1,77	23,12 ± 0,54	52,4 ± 1,73
0,9 % NaCl	-	19,3 ± 0,82	8,40 ± 0,14	28,8 ± 0,95
Heparin	-	37,6 ± 0,95	< 300	276,6 ± 9,15

Table 1. Anticoagulant activity of fucoid an depending on the intensity of ultrasound exposure and the presence of surfactants.

To determine the effect of polysaccharide samples on the external way of blood clotting, a PT test was performed. It was detected that this parameter increased with increasing ultrasound intensity. The mechanism of action of fucoidan in this test is based on thrombin fixation; therefore, the growth of PT to 33.4 ± 1.49 for processed fucoidan can be associated with a change in the electrochemical potential and the conformation of fucoidan molecules as a result of ultrasound processing, which facilitates its fixation with thrombin. When surface-active substances are administrated into the processing environment, the electrochemical potential of polysaccharide particles that exist in the solution may change, which changes the ability of fucoidan molecules to interact with thrombin. Prothrombin time increase was observed, with a value higher than that obtained for heparin as a result of PT test.

To detect the effect of fucoidan on the speed of fibrin generation from fibrinogen, a TT test was performed. The mechanism of effect of fucoidan on thrombin time is associated with inhibition of activity of a cascade of serine proteases[18] and interactions between thrombin and fibrinogen[19].

The speed of fibrine clot building *in vitro* in the presence of samples drops with increasing intensity of ultrasound up to 133 W / cm^2. This may be associated with a change in fucoidan gradesulfating[2] of fucoidan due to desulfating, oxidation, and other side effects of ultrasound that decrease the number of "available" sulfate groups.

With increasing intensity of ultrasound effect up to 200 W / cm^2 or with SAS administration with retained intensity of 133 W / cm^2, fibrinogen transformation to fibrin slows down, the maximum speed being 23.12 ± 0.54 s (when processed in an environment containing PEG - 400). On one hand, such change in TT test values may be associated with dropping size of fucoidan macro-molecules[20] as a result of ultrasound de-polymerization and with changed correlation between residual sugars and sulphate groups.[21] On the other hand, when ionogenic SAS is used in the processing environment, it may be absorbed on the surface of polysaccharide particles, changing their charge and conformation. These conditions allow fucoidan to be fixed more effectively with thrombin, while decreasing the functional effectiveness of enzymic complexes of blood clotting factors.

A similar change in activity can be seen when determining the effect of fucoidan on the internal way of blood clotting (APTT test). The highest value in this test, 52.8 ± 1.78 s, was obtained by the fucoidan sample generated with the maximum ultrasound intensity (200 W / cm^2).

Fucoidan inhibits biofilm formation in *Escherichia coli* and *Staphylococcus aureus*. It is known that crude fucoidans did not show antibacterial activity, but their depolymerized products inhibited the proliferation of *Escherichia coli* and Staphylococcus, which is confirmed in this work.[22]

Growth inhibition was enhanced with a decrease in the particle size of macromolecules (for samples 2 and 4, *Escherichia coli*; for 3 *Staphylococcus aureus*, compared to untreated fucoidan (Figure 1), which is consistent with the data obtained in.[23] This may be due to stronger polyanionic properties and conformational characteristics.[22,23]

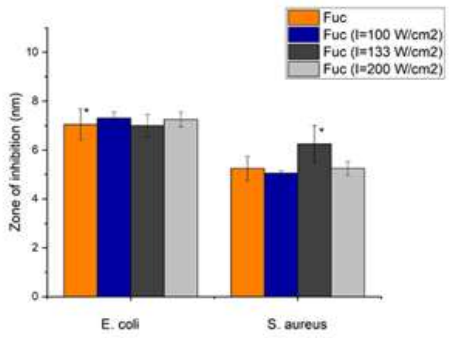

Figure 1. Disc diffusion assay shows the antibacterial efficacy of treatment fucoid an (*-results of 5 measurements)

In addition, the antibacterial action of fucoidan may be associated with the disruption of the cytoplasmic membrane permeability and resulting protein leakage and DNA damage.[14,24]

CONCLUSION

The effect of high intensity, low frequency ultrasound processing on the biological activity of fucoidan was detected. The study determined and selected the most favorable conditions for ultrasound processing (intensity, SAS) for polysaccharide destruction. We conclude that ultrasound processing of fucoidan is an environmentally friendly, inexpensive, and effective method for modifying this polymer; it can be used to obtain low-molecule fractions of fucoidan with predictable activity. During determination of methods of coagulation process inhibition, the effect of processed fucoidan on the external and internal ways of blood clotting was confirmed. Thanks to a lower anticoagulation activity of processed fucoidan vs. heparin, thrisk of bleeding can be reduced. Besides, low toxicity vs. heparin of animal origin may facilitate using the resulting fucoidan in the development of thrombolytic therapy products.

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