The Antithrombotic Effects of Low Molecular Weight Fragment from Enzymatically Modified of Laminaria Japonica Polysaccharide

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Source of support: Departmental sources

Background: Laminaria japonica polysaccharide (LJP), a fucose enriched sulfated polysaccharide has been demonstrated to have excellent anticoagulant and antithrombotic activities. However, the antithrombotic effect of low molecular weight polysaccharide from enzymatically modified of LJP (LMWEP) remains unknown.

Material/Methods: LMWEP was prepared by fucoidanase enzymatic hydrolysis, and the antithrombotic and anticoagulant activities, and the underlying mechanism were investigated thoroughly. Rats were randomly divided into 6 groups (8 rats in each group): the blank control group, the blank control group treated with LMWEP (20 mg/kg), the model group, the model group treated with heparin (2 mg/kg), the model group treated with LJP (20 mg/kg), and the model group treated with LMWEP (20 mg/kg). After 7 days of intravenous administration, blood was collected for biochemical parameters examinations.

Results: LMWEP increased the activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT), 6-keto prostaglandin F1α (6-Keto-PGF1α), and endothelial nitric oxide synthase (eNOS). In addition, LMWEP decreased fibrinogen (FIB), endothelin-1 (ET-1), thromboxane B2 (TXB2), erythrocyte sedimentation rate (ESR), and hematocrit (HCT).

Conclusions: LMWEP, an enzymatically modified fragment with a molecular weight of 25.8 kDa, is a potential antithrombotic candidate for treatment of thrombosis related diseases.

MeSH Keywords: Anticoagulants • Coronary Thrombosis • Embolism and Thrombosis

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/920221

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]
Background

Thrombosis is a multifactorial disease induced by facilitating a combination of hypercoagulability and stasis, and results in the formation of thrombus in a blood vessel [1]. Venous blood or arterial clots can cause cardiovascular event associated diseases, such as venous thromboembolisms, ischemic stroke, and ischemic myocardial infarction, and as a result can seriously impact quality of life and health quality [2]. It is generally known that thrombosis is associated with the activation of the extrinsic and intrinsic coagulation system, which can cause fibrin formation or aggregation of platelets [3]. Up until now, a lot of antithrombotic drugs, including anticoagulants and antiplatelets, have been used successfully for the treatment of thrombotic diseases, for a long time, and heparin is one of the most generally used antithrombotic drugs. However, these chemical drugs can cause many side effects and their clinical use is limited. Although low molecular weight heparins derived from heparin through specific depolymerization may have some advantages over original heparins in terms of reduced adverse effects and pharmacokinetics [4]. However, the yield limitation, structure variability, and safety issues arising from low molecular weight heparins of animal origin still are a concern [5]. Thus, it is of specific significance and urgency to search for novel antithrombotic agents from natural plants.

Laminaria japonica, a well-known marine brown alga in China, has been used as a valuable therapeutic agent for phlegm elimination, detumescence, and weight loss for several centuries [6]. Sulfated polysaccharides extracted from Laminaria japonica are commonly known as fucoids, which are mainly composed of fucose, galactose, and mannose. Pharmacology research has demonstrated that fucoids possess various biological activities, such as antitumor, anticoagulation, and antithrombotic activities, accompanied with activation of the fibrinolysis system and reduce hemorrhagic risk compared to heparin [7,8]. Previous research has demonstrated that the molecular weight, viscosity, and degree of fucose/sulfonation of polysaccharides have a great effect on their bioactivity [9–11]. In addition, it has been proven that the structure features of fucoidan influenced not only the antithrombotic and anticoagulant activities, but also the underlying mechanisms of these bioactivities. A previous study reported that the mechanism of fucoidan on antithrombotic effect was different from heparin. And high molecular weight fucoidan showed opposite activities on angiogenesis and platelet aggregation compared with lower molecular weight fucoidan [12]. In addition, molecular modification of the polysaccharide plays a vital role in enhancing bioactivities of polysaccharides [13]. Thus, the production of low molecular weight Laminaria japonica polysaccharide (LJP) is necessary to enhance antithrombotic activity. According to the literature, there are several ways to degrade high molecular weight polysaccharides, the enzymatic degradation has been proven to be an effective way to remove the glycosidic bonds of polysaccharides for the high selectivity, substrate specific and produce well-defined structures [14]. Previous literature has reported the antithrombotic activity of low molecular weight fucooidan from Laminaria japonica [15], and the antithrombotic activity of polysaccharide from Laminaria japonica has been reported [16]. However, there have been no literature reports of antithrombotic activity of the enzymatically modified Laminaria japonica polysaccharide (LMWEP), and the relationship between molecular weight and antithrombotic activity of LMWEP is still unknown. The main purpose of our present research was to explore these questions.

Thus, in this research, LJP was extracted from Laminaria japonica, and its LMWEP was prepared with fucoiodanase enzymatic degradation; the activities on platelet aggregation, arterial thrombosis formation, and blood circulation were assessed with the aim to provide pharmacological research to support clinical application. In addition, the possible underlying mechanism was also investigated to explore whether LJP fragments with different molecular weights acted on antithrombotic activity, and the relationship between molecular weight and bioactivities of polysaccharides were investigated.

Material and Methods

Reagents and animals

Laminaria japonica was collected on the coast of Rongcheng, Shandong, China in August 2018. The fresh algal material was washed, sundried, milled and then kept in plastic bags until further polysaccharide extraction. The fucoiodanase was produced as described in previous research [17]. Pullulan standards (0.342, 1.32, 12.6, 49.6, 218.2, 420.8, and 821.4 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat 6-keto prostaglandin Flx (6-Keto-PGF1α), thromboxane B2 (TXB2), endothelin-1 (ET-1), and endothelial nitric oxide synthase (eNOS) ELISA kits were purchased from Nanjing Senbeijia Biotech CO., Ltd. (Nanjing, China). All other chemical reagents with analytical grade were purchased from Aladdin (Shanghai, China).

The six-week-old male Sprague-Dawley rats (SCXK-2017-0012), weighing from 190 g to 210 g were purchased from the Experimental Animal Center of Hunan Province (Changsha, China) and were housed under maintained temperature (20±2°C), a relatively humidity of 45% to 60% (12 hours light/dark cycle) with freely access to water and standard rodent diet. The animal experiments were approved by the Animal Ethics Committee of Affiliated Hospital of Traditional Chinese Medicine of Southwest Medical University and conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.
Preparation of LJP and LMWEP

LJP was extracted from the dried Laminaria japonica (300 g) by hot water extraction based on previous literature with minor modification [18]. Briefly, the extraction parameter was: extraction temperature of 100°C, extraction time of 2 hours and water/solid of 12:1. After the extract was centrifuged, the supernatant was collected and condensed at 60°C under vacuum, and then added into 4 volume 80% ethanol at 2°C for 24 hours to precipitate polysaccharide. The crude polysaccharide was produced by centrifugation, lyophilized, and then deproteinized with Sevag method [19]. Then the crude polysaccharide was purified by using the dialysis membrane (cutoff 10 kDa) against pure water. Finally, the high molecular weight crude polysaccharide was lyophilized and labeled as LJP. The fucoidanase was selected to catalyze LJP depolymerization under the condition of 37°C, pH 7.2, and incubation for 7 hours and thereafter deproteinized by heating at 100°C for 10 minutes; the precipitate was removed by centrifugation. Finally, the supernatant was purified with the dialysis membrane (cut-off 10 kDa) against pure water and thereafter lyophilized, labeled as LMWEP.

Experimental model and drug treatment

Forty-eight Sprague-Dawley rats were randomly divided into 6 groups (8 rats in each group): the blank control group (NC), NC group treatment with LMWEP (NC+LMWEP), model group (MC), heparin model group (MC+heparin) and 2 model treated group (MC+LJP and MC+LMWEP). The rats of the NC group and the MC group were given the same saline by intravenous administration. The heparin group was given 2 mg/kg heparin. The 2 treated groups were given LJP (20 mg/kg) and LMWEP (20 mg/kg), respectively. The dose of LJP was based on a previous report [20]. All treatments were carried out by intravenous administration daily for 7 days.

After the fifth intravenous administration, the acute blood stasis model was established, expect for the NC rats. All model rats were subcutaneously injected with 0.8 mg/kg adrenaline hydrochloride. The rats were placed in ice-cold water for 5 minutes after 2 hours of the first subcutaneously injection with adrenaline hydrochloride, and then subcutaneously injected with 0.8 mg/kg adrenaline hydrochloride again 2 hours after the ice-cold bath to establish blood stasis model [21]. All rats were fasted overnight with free access to water and treatment continued after establishing the model. After 18 hours of the last subcutaneously injection of adrenaline hydrochloride, all rats were anesthetized with 300 mg/kg 10% chloral hydrate with 30 minutes after the last treatment, then the blood was collected from the abdominal aortas to measure the biochemical parameters.

Anticoagulation assay in vivo

Activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT), and fibrinogen (FIB) were measured to examine whether the LMWEP had effects on the coagulation in blood stasis rats. The blood samples were collected into centrifuge tube mixed with 3.8% sodium citrate (citrate/blood: 1/9, v/v) and then plasma was obtained by centrifugation of 4000 rpm for 10 minutes at 5°C. APTT, PT, TT, and FIB content were determined using Automated Coagulation Analyzer (CA-7000, Sysmex, Japan).

Determination of hemorheology parameters

The whole blood viscosity (WBV) of blood samples were determined by the Auto Viscometer (LBY-N6B, Precil Instrument Co., Ltd., China). The blood samples were collected into vacuum tube mixed with 3.8% sodium citrate (citrate/blood: 1/9, v/v) for the measurement of WBV, including high shear rate (200/s), middle shear rate (30/s) and low shear rate (3/s). Erythrocyte sedimentation rate (ESR) value was measured by Westergren method and hematocrit (HCT) value was determined by the Wintrobe method.

Determination of 6-Keto-PGF1α, ET-6, eNOS, and TXB2

To investigate the possible mechanism in vivo, the plasma levels of 6-Keto-PGF1α, ET-6, eNOS, and TXB2 were measured. The blood samples were collected into centrifuge tube mixed with 3.8% sodium citrate (citrate/blood: 1/9, v/v), centrifuged at 4000 rpm for 10 minutes to separate plasma. The plasma was obtained to determine the plasma levels of 6-Keto-PGF1α, ET-1, eNOS and TXB2. All plasma parameters were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s procedures.

Determination of platelet aggregation

To evaluate the effect of LMWEP on platelet aggregation, we assayed the suppression of LMWEP on platelet aggregation in blood stasis rats. The blood samples were collected into centrifuge tube mixed with 3.8% sodium citrate (citrate/blood: 1/9, v/v). Platelet rich plasma (PRP) was prepared by centrifugation of 1000 rpm for 8 minutes. Then the PRP was removed, and the remaining blood samples were centrifuged at 3000 rpm for 15 minutes to obtain platelet poor plasma (PPP). Then, 0.3 mL of PRP was added into a cuvette, after which 6 μM ADP (adenosine diphosphate) was added. Platelet aggregation was assayed using the platelet aggregometer. Data are expressed as percentage of maximal aggregation.
HPSEC-RI measurement

The weight-average molecular weights of polysaccharide fractions were determined using a high-performance size exclusion chromatography (HPSEC) (Waters 2695, USA) combined with a refractive index detector. HPSEC-RI was carried out with a Shodex-OHpak SB-804 HQ column (8.0×300 mm), all samples were dissolved in 0.1% sodium chloride, the mobile phase was 0.1% sodium chloride aqueous solution at 35°C with a flow rate 1 mL/minute and the injection volume was 0.1 mL. The molecular weight of the polysaccharide fraction was calculated using the pullulan standard curve. The chromatography of pullulan standards and sample was consistent.

Toxicity study

Serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum urea nitrogen (BUN), and serum creatinine (CRE) were assayed using an ELISA kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's procedures.

Statistical analysis

Experimental results were displayed using the mean±standard deviation (SD). Statistical comparison between any 2 groups was utilized by the paired t-test. All data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). P-values of < 0.05 were defined as statistically significant.

Results

Total polysaccharides and molecular weight of LJP and LMWEP

As displayed in Table 1, the total polysaccharides contents of LJP and LMWEP determined by phenol-sulfuric acid method were 67.63% and 65.35%, respectively. Meanwhile, the molecular weight of LJP and LMWEP were determined by HPSEC-RI. The molecular weight of LJP was 104.5 kDa, which was higher than LMWEP (25.8 kDa). As showed in Figure 1, after enzymatic hydrolysis by fucoidanase, the peak retention time of LJP was shorter than LMWEP. These results implied that the LJP was enzymatically degraded into low molecular weight polysaccharide.

The effects of LJP and LMWEP on WBV in vivo

As shown in Table 2, when compared with the NC group, the WBV was obviously increased at all shear rates in the MC group (P<0.01), indicating that the blood stasis model was established successfully. However, intravenous administration of...
LMWEP and LJP obviously decreased WBV at all shear rates compared with the MC group ($P<0.01$, $P<0.05$) and the effect on WBV of LMWEP at all shear rates was equal to heparin ($P>0.05$). Meanwhile, compared to the LJP group, the WBV at all shear rates of the LMWEP group was even lower ($P<0.05$).

**Effect on plasma coagulation system in vivo**

Effects of LJP and LMWEP on coagulation system (APTT, TT, PT, and FIB) are displayed in Figure 2. APTT, PT, and TT were obviously decreased, and the content of FIB was significantly increased in the MC group compared with the NC group ($P<0.01$). Apparently, intravenous administration of LJP or LMWEP effectively prolonged the APTT, PT, and TT and decreased the FIB content compared with the MC group ($P<0.01$, $P<0.05$), especially LMWEP group.

**Effect on the plasma levels of ET-1 and eNOS in vivo**

As displayed in Figure 3, the plasma eNOS levels in MC rats was obviously lower than that of the NC group rats ($P<0.01$), whereas the plasma ET-1 levels in MC rats was significantly higher than that of the NC group ($P<0.01$). Apparently, intravenous administration of LMWEP effectively upregulates plasma eNOS levels and downregulates plasma ET-1 levels compared with the MC model group ($P<0.01$). However, compared with the MC group, LJP only declined in plasma ET-1 levels ($P<0.05$) and showed no effect on plasma eNOS levels.

**Effect on the plasma levels of 6-Keto-PGF1α, TXB2, and platelet aggregation rate in vivo**

The effects of different molecular weight polysaccharide fractions on 6-Keto-PGF1α and TXB2 levels, ratio of TXB2/6-Keto-PGF1α, and platelet aggregation in stasis rats are shown in Figure 4. Our research results showed that subcutaneous injection of adrenaline hydrochloride caused obviously decrease of plasma 6-Keto-PGF1α levels and increase of plasma TXB2 levels in the MC group, accompanied with significantly increase of TXB2/6-Keto-PGF1α and platelet aggregation. There was an interesting finding that LJP only declined plasma TXB2 levels and showed no effect on plasma 6-Keto-PGF1α levels and platelet aggregation. However, intravenous administration of LMWEP obviously increased plasma 6-Keto-PGF1α.
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Figure 3. The effect of LMWEP treatment on the plasma levels of ET-1 (A) and eNOS (B) in thrombosis rats. The data are reported as the mean±SD of 8 rats per group. ** P<0.01 (versus NC group) and *** P<0.01, * P<0.05 (versus MC group). LMWEP – low molecular weight enzymatic Laminaria japonica polysaccharides; ET-1 – endothelin-1; eNOS – endothelial nitric oxide synthase; SD – standard deviation; NC – normal control, MC – model control.

Figure 4. The effect of LMWEP treatment on the plasma levels of TXB2 (A), 6-Keto-PGF1α (B), ratio of TXB2/6-Keto-PGF1α (C) and platelet aggregation (D) in thrombosis rats. The data are reported as the mean±SD of 8 rats per group. ** P<0.01 (versus NC group) and *** P<0.01, * P<0.05 (versus MC group). LMWEP – low molecular weight enzymatic Laminaria japonica polysaccharides; TXB2 – thromboxane B2; Keto-PGF1α – 6-keto prostaglandin F1α; SD – standard deviation; NC – normal control, MC – model control.
Figure 5. The effect of LMWEP administered by intravenous routine on ESR (A), HCT (B) in thrombosis rats. The data are reported as the mean±SD of 8 rats per group. ** P<0.01 (versus NC group) and *** P<0.01, * P<0.05 (versus MC group). LMWEP – low molecular weight enzymatic Laminaria japonica polysaccharides; ESR – erythrocyte sedimentation rate; HCT – and hematocrit; SD – standard deviation; NC – normal control, MC – model control.

Figure 6. Effect of LMWEP on serum biochemical parameters in thrombosis rats. The data are reported as the mean±SD of 8 rats per group. LMWEP – low molecular weight enzymatic Laminaria japonica polysaccharides; SD – standard deviation.

levels and suppressed plasma TXB2 levels in thrombosis rats, accompanied with the decline of TXB2/6-Keto-PGF1α and platelet aggregation.

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Effects on hemorheology in vivo

Figure 5A and 5B shows the different molecular weight polysaccharide fractions on hemorheology in thrombosis rats. Subcutaneously injection of adrenaline hydrochloride caused an obviously increase of HCT and ESR values, compared with the NC group (P<0.01), implying that the acute blood stasis
In our research, the anticoagulant activity of LJP and LMWEP are concerned with thrombin-medicated fibrin formation [28]. Previous literature reports [27]. PT and APTT reflect extrinsic coagulation pathways, and the suppression of fibrin formation. Hemorheology is concerned with blood pressure and flow, constriction of blood vessels, and flow volume, such as measured by WBV, ESR, and HCT. The hemorheology measurement has been commonly used in the clinical setting, especially for prevention and diagnosis of cardiovascular and thromboembolic diseases [29,30]. ESR also reflects red blood cell aggregation and whole blood viscosity. HCT is the important factor of WBV [31]. In this study, LJP and LMWEP could obviously decline WBV at various shear rates, as well as ESR and HCT values, which further indicated an antithrombotic effect of LJP and LMWEP.

Discussion

It is well known that low molecular weight heparin is a good alternative drug for treatment of thromboembolism disease, since it possesses a better bioavailability on the intravenous administration and little variability in coagulation response [22,23]. It is also known that the sulfate group of polysaccharide plays a vital role in anticoagulant and antithrombotic properties [24]. We should search for the ideal antithrombotic agent from the non-animal source to avoid the risk of products that contain animal pathogens. LJP is a native sulfated polysaccharide, mainly compose of fucose, galactose, mannose, and rhamnose. It also contains glucuronic and sulfated ester. LJP exhibits prominent antithrombotic and anticoagulant properties, and the anticoagulant effect of sulfated polysaccharides has been related to their sulfur content, molecular weight, and type of monosaccharides, as well as other factors [25]. Because the LJP with its high molecular weight has poor solubility and high hemorrhagic risk, the LMWEP fraction was obtained by fucoidanase enzymatic hydrolysis. The molecular weight of LMWEP (25.8 kDa) was lower than that of LJP (104.5 kDa). The sulfated ester of LMWEP and LJP were 33.89% and 28.36%, respectively, indicating that the sulfated ester was not removed from the LJP backbones and the molecular weight of LJP was obviously decreased after enzymatic hydrolysis. The present research aimed to demonstrate the antithrombotic action of LJP and its modified forms of LMWEP on blood stasis rats.

According to traditional Chinese medicine theory, low temperature accumulation could induce blood stasis through decreasing the blood flow rate. Adrenaline hydrochloride could give rise to hemorheological disorders by increasing whole blood viscosity and constricting blood vessels [26]. The blood stasis model in this study was established using ice water after subcutaneously injection of adrenaline hydrochloride according to previous literature reports [27]. PT and APTT reflect extrinsic and intrinsic coagulation pathways, respectively. FIB and TT are concerned with thrombin-mediated fibrin formation [28]. In our research, the anticoagulant activity of LJP and LMWEP from Laminaria japonica was investigated in vivo. The LJP and LMWEP groups obviously extended APTT, PT, and TT and decreased the content of FIB in vivo, which showed a positive effect on extrinsic and intrinsic coagulation pathways, and the suppression of fibrin formation.

Toxicological effects of LJP and LMWEP

As shown in Figure 6, when compared with the NC group, the administration of LJP or LMWEP had no apparent effect on renal function and hepatic function indicators, indicating that the treatment of LJP or LMWEP possessed no toxicological effect in blood stasis model.

Additionally, it has been shown that the molecular weight of sulfated polysaccharides has a great effect on the antithrombotic, anticoagulation, and platelet aggregation effects [37,38]. A previous study [39] demonstrated that low molecular weight fragments including dFCSc and dFCSt exhibited superior antithrombotic-hemorrhagic ratios than native polysaccharide, which was in agreement with our present research, where we found that LMWEP possessed superior antithrombotic and antiplatelet activities than the native polysaccharide.
Conclusions

LMWEP was prepared by fucoidanase enzymatic hydrolysis. Our findings revealed that LMWEP possessed superior anti-coagulant, antithrombotic, and antiplatelet activities than the native polysaccharide. Furthermore, the antithrombotic action of LMWEP could be associated with its protective effect of vascular endothelium, inhibition of platelet aggregation, and maintenance of the balance of TXB2 and 6-Keto-PGF1α. Thus, LMWEP could become a potential antithrombotic candidate for further research.

Conflicts of interest

None.

References: