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Articles

Isolation and Partial Characterization of a Polysaccharide with Antithrombin Activity against Blood Coagulation in Manda®, a Fermented Natural Food

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A polysaccharide with antithrombin activity in Manda® (PAM) was purified via procedures comprising three major steps, i.e. fractional precipitation with ethanol, anion exchange chromatography, and gel permeation chromatography. PAM showed a symmetrical peak on size exclusion HPLC, as assessed by refractive index, and behaved as a single band on cellulose acetate electrophoresis. The average molecular mass was estimated to be 222 kDa by gel filtration. PAM was found to be a sulfated heteropolysaccharide that contains sulfate group (20.5%, w/w) and uronic acid moiety (7.1%, w/w) in addition to neutral sugar consisting of fucose, xylose, mannose, galactose, and glucose in a molar ratio of 1.00 : 0.35 : 0.28 : 0.22 : 0.15. This polysaccharide appeared to inhibit blood coagulation via the intrinsic pathway in a dose-dependent pattern. The clotting of fibrinogen by thrombin was also significantly mitigated by the presence of PAM.

Key words: *Manda, sulfated polysaccharide, anticoagulant, antithrombin activity.*

Whenever a blood vessel is severed or ruptured, both the intrinsic and extrinsic pathways of the coagulation cascade may be activated, and thrombin is ultimately generated from its zymogen during the blood coagulation process. Thrombin acts as an enzyme to convert soluble fibrinogen into fibrin threads that enmesh platelets, blood cells, and plasma to form a clot.[1)] Thrombin activates other hemocoagulant enzymes including factors V, VIII, and XIII[2)] and is also a potent platelet activator, thus promoting platelet aggregation.[3)] These processes may lead to thrombosis, a pathological phenomenon resulting from the excessive formation of platelet/fibrin rich thrombi in the arterial tree. Therefore, thrombin inhibition would provide an effective means of controlling thrombosis. Heparin, the best known among thrombin inhibitors, has long been used clinically to prevent the recurrence of venous thromboembolism.[4)] Due to undesirable side effects of heparin, such as bleeding, thrombocytopenia, and osteoporosis, many attempts have been made to find new anticoagulant and antithrombin compounds for the development of antithrombotic drugs. Except for hirudin that is a single chain polypeptide, anticoagulant and antithrombin activities have mostly been found in numerous sulfated polysaccharides including fucoidans from brown seaweeds.[5-14)]

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Abbreviations: PAM, polysaccharide with antithrombin activity in Manda®; aPTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time.

Varieties of fermented foods have been claimed to be physiologically functional; one of these is Manda®, which is manufactured via yeast fermentation of mixtures of various vegetables, fruits, and seaweeds. The antioxidant and antitumor effects and the suppression effect against lipid peroxidation in the senescent rat brain seem to be in support of the claim for Manda®.[15,16)] Having observed in the preliminary experiment that the aqueous extract of Manda® has a substantial blood coagulation-inhibiting activity, in the present work we isolated and partially characterized a sulfated polysaccharide with antithrombin activity against fibrinogen clotting from Manda®, which is hereafter referred to as PAM.

Materials and Methods

Materials. Manda® was obtained from Manda® Fermentation (Hiroshima, Japan). Heparin from porcine intestinal mucosa, fucoidan from *Fucus vesiculosus*, human thrombin, and bovine fibrinogen were purchased from Sigma Chemical (St. Louis, MO). Citrated pooled human plasma, actin-activated cephaloplastin reagent, and thromboplastin C plus were purchased from Dade International (Miami, FL). DEAE-Sepharose CL-6B and Sepharose CL-6B were obtained from Pharmacia Biotech (Uppsala, Sweden). Chromozym TH (tosyl-Gly-Pro-Arg-*p*-nitroanilide) was

from Boehringer Mannheim (Basle, Switzerland). Cellulose acetate membrane was purchased from Helena Laboratories (Beumont, Tx).

Purification of a polysaccharide with antithrombin activity. Manda® (150 g-wet weight) was suspended in 300 m*l* distilled water, stirred for 2 h at room temperature, filtrated through three layers of cheese cloth, and centrifuged at 11,000 × g for 20 min. Cold ethanol was added to the supernatant to make a final concentration of 10% (w/v). The solution was stirred at room temperature for 20 min and centrifuged. The pellet was then discarded. The supernatant was brought to 55% (w/v) ethanol concentration with cold ethanol and treated as above except that the pellet was retained. The pellet was dried in N_2 stream and dissolved in 400 m*l* of 50 mM Tris buffer (pH 7.5) containing 0.3 M NaCl. The solution was then centrifuged, and the precipitate was discarded. The soluble polysaccharides (1.5 g) were loaded onto a DEAE-Sepharose column (6.7 cm × 2.5 cm) equilibrated with 50 mM Tris buffer (pH 7.5) containing 0.3 M NaCl and washed with 400 m*l* of the same buffer. The elution was done with a linear gradient of NaCl (0.3-1.0 M) in 50 mM Tris buffer (pH 7.5) at a flow rate of 60 m*l*/h. The active fractions with antithrombin activity were pooled and dialyzed against distilled water. The partially purified polysaccharide preparation (61 mg) was loaded onto a Sepharose CL-6B column (115 cm × 1.28 cm) and eluted at a flow rate of 30 m*l*/h with 50 mM Tris buffer (pH 7.5) containing 0.5 M NaCl. The fractions of PAM were pooled, dialyzed against distilled water, and lyophilized (24 mg).

Size exclusion HPLC and electrophoresis. Size exclusion HPLC with PAM was carried out on a Shodex OHpack KB-805 column (Showa Denko, Tokyo, Japan) which separates dextrans with molecular masses that range approximately from 10 to 2,000 kDa. Electrophoresis was performed using a method similar to that of Nardella *et al.*[17] on a cellulose acetate membrane. After electrophoretic run in 0.3 M calcium acetate (pH 7.5), the membrane was stained with 0.1% Toluidine blue in 3%(w/v) acetic acid and then destained with 3% acetic acid. A commercial fucoidan from *Fucus vesiculosus* was used as the standard.

Determination of molecular weight. The average molecular weight of PAM was estimated as in Zhuang *et al.*[18] using a Sepharose CL-6B gel filtration column equilibrated with 0.3 M NaCl. A calibration curve was obtained with dextrans (MW 464, 282, 148 and 67 kDa) as molecular weight markers.

Chemical analyses. Total sugar content was determined using the phenol-sulfuric acid method of Dubios *et al.*[19] with fucose as the standard. Uronic acid was measured using the modified *m*-hydroxydiphenyl-sulfuric acid method of Blumenkrantz and Asboe-Hansen[20] with D-glucuronic acid as the standard. Sulfate content was determined essentially according to Dogson and Price[21] with K_2SO_4 as the standard for the turbidimetric method. IR spectra were measured with a Bio-Rad FTS-60 spectrometer (Hercules, CA). Neutral sugars were analyzed as described by Jones and Albersheim.[22] PAM (2 mg) was hydrolyzed with 2 M trifluoroacetic acid at 121°C for 1.5 h, and the alditol acetate derivatives of sugars were measured in a Young-Lin M600D gas chromatograph (Seoul, Korea) equipped with a Supelco SP-2380 column (Supelco, Bellefonte, PA). Myo-inositol was used as an internal standard.

Assays for plasma coagulation. The activated partial thromboplastin time (aPTT), prothrombin time (PT), and thrombin time (TT) were measured through the procedures adopted from Fox *et al.*[23] using a blood coagulation analyzer (model; Coag-Stat™, Kyoto Daichi, Kyoto, Japan). A mixture of PAM solution (15 μ*l*, 0 25 μg/m*l*), citrated pooled human plasma (100 μ*l*), and aPTT reagent (100 μ*l*) was incubated for 3 min at 37°C, and aPTT was measured after the addition of 100 μ*l* of 20 mM $CaCl_2$ to the mixture. Citrated pooled human plasma (100 μ*l*) was incubated with PAM solution (15 μ*l*, 0-25 μg/m*l*) for 3 min at 37°C, and PT and TT were measured after the addition of 200 μ*l* of thromboplastin C reagent and 100 μ*l* of thrombin solution (5 U/m*l*), respectively.

Assays for thrombin activity. Thrombin activity was measured as described by Cappiello *et al.*[24] Fibrinogen clotting was assayed as follows. Human plasma thrombin and bovine plasma fibrinogen were respectively dissolved in a reaction medium consisting of 100 mM NaCl, 10 mM $CaCl_2$, and 0.1% PEG6000 in 10 mM Hepes/10 mM Tris buffer (pH 7.4). PAM at various concentrations (10 μ*l*, 0-24 μg/m*l*) in distilled water was mixed with 0.125% (w/v) fibrinogen solution (200 μ*l*) and preincubated for 3 min at 37°C. Thrombin (5 U/m*l*, 100 μ*l*) was added to the mixture, and the clot formation time was measured using the blood coagulation analyzer. Amidolytic activity was measured as follows. Human plasma thrombin dissoved in the reaction medium was incubated with the polysaccharide for 3 min at 25°C. Amidolytic reaction was initiated by the addition of Chromozym TH (1.9 mM) to the incubated mixture, and thrombin-catalyzed hydrolysis of Chromozym TH was measured by spectrophotometrically monitoring the release of p-nitroaniline at 405 nm.

Results and Discussion

PAM was purified by a combination of ethanol fractionation, anion exchange chromatography, and gel filtration chromatography. The ion exchange chromatography separated the antithrombin active materials of Manda® into two fractions. Since the minor fraction showed very low activity, further purification was performed only with the major fraction in a Sepharose CL-6B column, which gave rise to a chromatogram with a single and virtually symmetrical band of polysaccharide showing antithrombin activity (Fig. 1).

Cellulose acetate electrophoresis revealed that the polysaccharide preparation behaved as a single band, and the

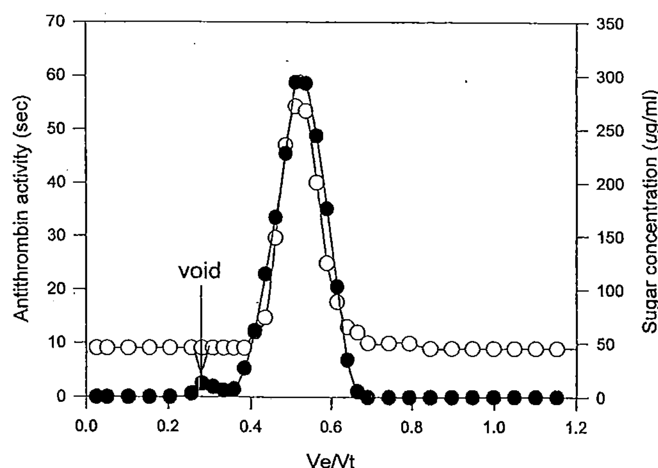

Fig. 1. Purification of the polysaccharide with antithrombin activity by Sepharose CL-6B gel filtration chromatography as the final step. The antithrombin activity was measured by an increase in fibrin clotting time, and total sugar content was determined by the phenol-sulfuric acid method. Open circles, antithrombin activity; Closed circles, total sugar concentration.

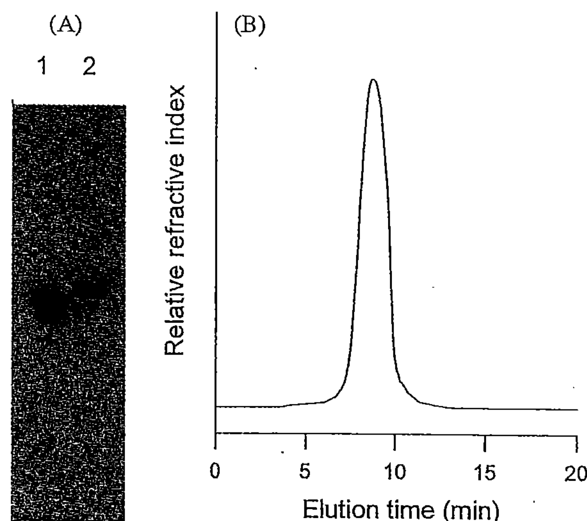

Fig. 2. Electrophoresis and Size exclusion HPLC of PAM. (A) Electrophoresis was run on a cellulose acetate membrane in 0.3 M calcium acetate (pH 7.5) for 1.5 hr at 1.5 mA/cm. Lane 1; a commercial fucoidan from *Fucus vesiculosus*, Lane 2; PAM. (B) Size exclusion chromatography was carried out using a Shodex OHpack KB-805 column in 0.2 M NaCl at the flow rate of 1.0 m*l*/min.

size exclusion HPLC gave a rather sharp symmetrical peak, as assessed by refractive index (Fig. 2). These results indicated that PAM was purified nearly, if not completely, to homogeneity. The average molecular mass of PAM was estimated to be 222 kDa via Sepharose CL-6B gel filtration using dextrans as standards (data not shown). The average molecular weight of PAM was different from those of active sulfated polysaccharides from various sources.[6-10, 25)]

PAM was found to be a sulfated heteropolysaccharide, containing substantial amounts of sulfate group (20.5%, w/w) and uronic acid (7.1%, w/w), as summerized in Table 1. Neutral sugar of PAM consists of fucose, xylose, mannose, galactose, and glucose, among which fucose is the major one, comprising 50% of the total neutral sugar units. Although numerous fucose-containing sulfated polysaccharides with anticoagulant and antithrombin activities have been isolated from a mushroom and several seaweeds, the sulfate content and sugar composition of PAM are apparently distinctive from those of other active fucoidan sulfates.[6-13, 25)] Measurement of IR spectroscopic property of PAM revealed that there are at least two absorption bands characteristic of sulfate group attached to sugar moiety. In addition to the strong band of S=O stretching at 1257 cm^{-1}, an absorption peak that is believed to arise from C-O-S appeared at 847 cm^{-1} (Fig. 3). This IR peak indicates that the binding of sulfate group to fucopyranose occurs at the axial C-4 position in the C1 conformation[6)]. The IR spectrum of PAM was strikingly similar to that of an anticoagulant fucoidan sulfate from *Fucus vesiculosus*[6)] with respect to the general shape as well as the peak positions.

The anticoagulant activity of PAM was examined with human plasma using aPTT, PT and TT assays. The anticoagulant action was seen in aPTT and TT in a dose-dependent manner but not in PT, suggesting that the PAM activity may be related to the intrinsic coagulation pathway and the third coagulation phase in the plasma[26)] (Fig. 4). The

Table 1. Comparison of physicochemical properties and anticoagulant activity of sulfated polysaccharides from various sources.

Sources	MW (kDa)	Sulfate (%)	Neutral sugar composition (molar ratio)					Activity[3] (units/mg)	Ref.
			Fuc	Xyl	Man	Gal	Glc		
F. vesiculosus[1]	100 100	10.2 7.6	1.00 1.00	0.00 0.12	0.00 0.10	0.16 0.09	0.00 0.00	-	6
L. angustata Var. *longissima*[1]	400	38.4	1.00	0.00	0.00	46.58	0.00	16	9
P. pavonia[1]		18.6	1.00	1.00	0.80	0.80	0.67	-	12
C. versicolor[2]	720	19.3	1.00	~0.00	0.22	0.11	0.20	1.1	25
Manda®	222	20.5	1.00	0.35	0.28	0.22	0.15	11.1	This work

[1]Brown seaweed
[2]Mushroom
[3]Anticoagulant activity measured by aPTT assay is given. Heparin showed anticoagulant activity of 172±5 units/mg in the same assay system.

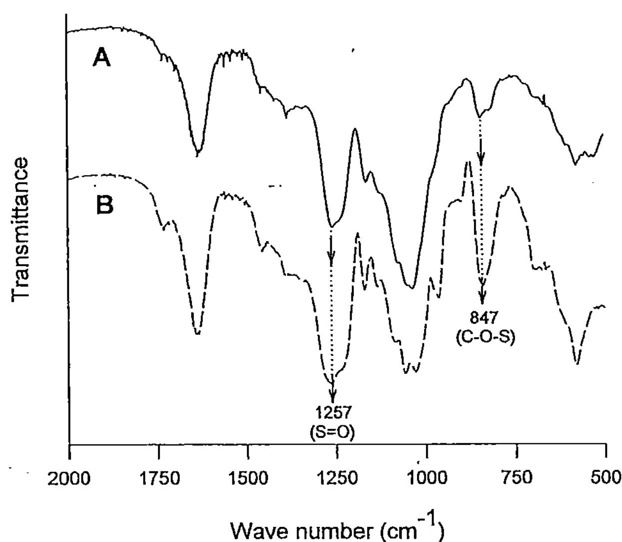

Fig. 3. IR spectrum of PAM (A) compared with that of a commercial fucoidan from *Fucus vesiculosus* (B).

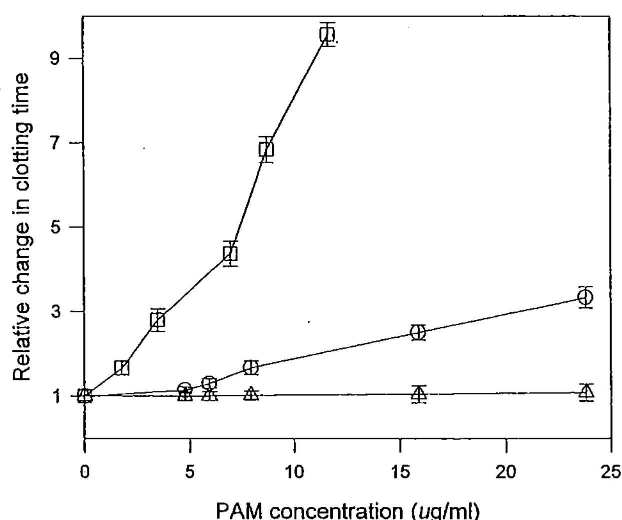

Fig. 4. Effects of PAM on the process of plasma coagulation. Data for changes in TT (squares), aPTT (circles) and PT (triangles) with increasing concentrations of PAM in the respective reaction mixtures were means of triplicated experiments with error bars indicating SD.

marked increase in TT, in particular, indicated that the third coagulation phase related to the thrombin-mediated fibrin formation was severely inhibited. The doubling of clotting time in aPTT and TT assays occurred at PAM concentrations of 14.3 and 2.4 μg/m*l*, respectively, comparable to the concentration range for *in vivo* experiments with anticoagulant substances.[27]

The effects of PAM on fibrin formation and amidolytic reaction by thrombin were examined (Fig.5). PAM significantly inhibited thrombin-catalyzed fibrinogen clotting in the absence of protease inhibitors such as heparin cofactor II and antithrombin III. A 50% decrease in the clotting was seen at PAM concentration of 0.29 μg/m*l*. However, PAM at concentrations of up to 5 μg/m*l* did not

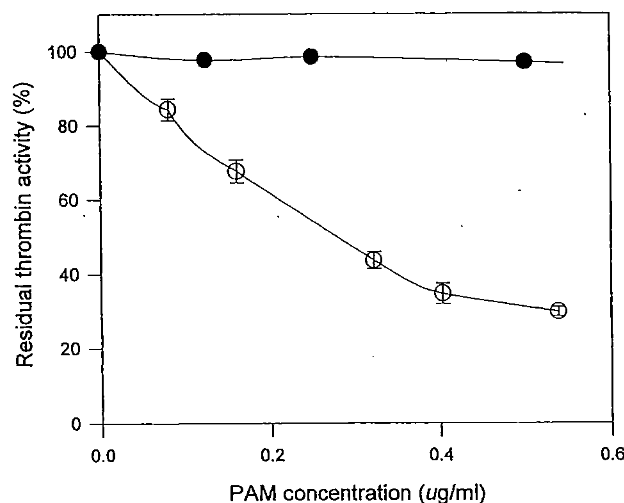

Fig. 5. Fibrinogen clotting activity (open circles) and amidolytic activity (closed circles) of thrombin as functions of PAM concentration in the respective reaction mixtures. Data are presented as mean±SD (n=3).

affect the amydolytic activity of thrombin. Even at higher concentrations (>50 μg/m*l*), PAM inhibited the amydolytic activity only slightly, by about 7% (data not shown). A similar observation has been reported; a hirudin-derived synthetic peptide inhibits fibrinogen-clotting activity of thrombin but fails to exert any effect on amidolysis of Chromozym TH.[5] There may be two plausible explanations for such effect of antithrombin substances on fibrinogen-thrombin interaction. First, the active substances bind to thrombin at or near the catalytic site, blocking the approach of fibrinogen to thrombin. Second, they bind to fibrinogen at or near the thrombin-attacking site, blocking the approach of thrombin to fibrinogen. The relevant data obtained with PAM seem to be pertinent to the latter explanation because the amidolytic activity of thrombin *per se* remained intact in the presence of relatively high concentrations of PAM while the fibrinogen-clotting activity was remarkably decreased by low concentrations of PAM. In line with this view, the inhibitory effect of an active fucoidan on fibrinogen-thrombin interaction has been attributed to the steric hindrance arising from the polysaccharide binding to fibrinogen.[14, 28]

In conclusion, we consider that PAM is a new polysaccharide with anticoagulant and antithrombin activities, which is apparently different in molecular weight and sugar composition from other active fucoidan sulfates previously reported. At present, however, no information is available as to whether PAM is present in the raw materials of Manda® manufacture or originates from the yeast fermentation. This is a current subject of experimental scrutiny in our laboratory.

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