J. Ginseng Res.
Vol. 23, No. 4, 217~221(1999)

Antithrombin Active Polysaccharide Isolated from the Alkaline Extract of Red Ginseng

Dong Chung Kim, Man-Jin In*, Ji Young Lee, Yoon Kyung Hwang** and Sung Dong Lee#

Korea Nutrition Research Institute, Korea University, Seoul 136-701, Korea
**Department of Human Nutrition and Food Science, ChungWoon University, Chungnam 350-800, Korea*
*** Department of Food and Culinary Art, Suwon Womens College, Suwon 441-748, Korea*
#Department of Food and Nutrition, College of Health Science, Korea University, Seoul 136-703, Korea
(Received December 22, 1999)

Abstract : We have isolated an antithrombin active polysaccharide in red ginseng by procedures comprising three major steps involving alkaline extraction, anion exchange and gel permeation chromatography. Active polysaccharide behaved as a single band on cellulose acetate membrane electrophoresis. The average molecular mass was estimated to be about 177 kDa by gel filtration. This polysaccharide was found to be an acidic heteropolysaccharide that contains uronic acid moiety (40.2%), sulfate group (9.2%) and protein (1.5%) in addition to neutral sugar consisted of rhamnose, mannose, galactose, arabinose, glucose, fucose and xylose in a molar ratio of 1.00 : 0.88 : 0.86 : 0.78 : 0.70 : 0.33 : 0.22. This polysaccharide inhibited blood coagulation via the intrinsic pathway like heparin in a dose-dependent manner. The clotting of fibrinogen by thrombin was also mitigated by the presence of this polysaccharide.

Key words : Red ginseng, acidic polysaccharide, anticoagulant, antithrombin activity

Introduction

Whenever a blood vessel is injured, both the intrinsic and extrinsic pathways of the coagulation cascade are activated and soluble fibrinogen is ultimately converted into fibrin threads that enmesh platelets, blood cells and plasma to form the clot.[1] However, abnormality of this process may lead to thrombosis, a pathological phenomenon resulting from the excessive formation of platelet/fibrin rich thrombi in the arterial tree. Therefore, many attempts have been made to find anticoagulant and antithrombin active compounds aiming at developing antithrombotic drugs. These activities have mostly been found in numerous sulfated polysaccharides including heparin[2] and fucoidans.[3-5] Recently, anticoagulant active polysaccharides were reported from several plants of mushroom[6] and brown seaweed.[7-8]

Korean ginseng, *Panax ginseng* C.A. Meyer, has been known as one of the important herbal medicines for more than 2,000 years in oriental countries. It has been reported that ginseng has a wide range of pharmacological properties including antifatique, antiinflammatory, antistress and antitumor action.[9-11] One of the most extensively studied component of ginseng is acidic polysaccharides. Acidic polysaccharides of (red) ginseng have been reported to possess antitumor, immunomodulating, hypoglycemic and anticomplementary activity.[12-14] However, there have not been reports on anticoagulant or antithrombin activity of acidic polysaccharides of ginseng.

Having observed in the preliminary experiment that the aqueous alkaline extract of red ginseng has a substantial inhibitory activity of fibrin clotting, in the present work we isolated and partially characterized an acidic polysaccharide with bio-activity against fibrinogen clotting and blood coagulation from red ginseng.

#To whom correspondence should be addressed.
(Tel) +82-2-940-2851; (Fax) +82-2-915-5704
E-mail: lsdojm@hanmail.net

Experimental

1. Materials

Red ginseng was obtained from Korea Tobacco and Ginseng Cooperation (Taejoen, Korea). Human thrombin and bovine fibrinogen were purchased from Sigma Chemical (St. Louis, MO). Citrated pooled human plasma, actin-activated cephaloplastin reagent, thrombin reagent and thromboplastin C plus reagent were from Dade International (Miami, FL). DEAE-Sepharose CL-6B and Sepharose CL-6B were obtained from Pharmacia Biotech (Uppsala, Sweden). Cellulose acetate membrane was purchased from Helena Laboratories (Beumont, Tex).

2. Assay for antithrombin activity

Antithrombin activity was measured as in Cappiello *et al.*[15] Human plasma thrombin and bovine plasma fibrinogen were respectively dissolved in 50 mM Tris buffer (pH 7.4) containing 50 mM NaCl. The sample preparations (10 μl) at various concentrations in distilled water were mixed with 0.125% (w/v) fibrinogen solution (200 μl) and preincubated for 3 min at 37°C. Thrombin (5 U/ml, 100 μl) was added to the mixture and then the clot formation time was measured using the blood coagulation analyzer (model; Coag-Stat™, Kyoto Daichi, Kyoto, Japan).

3. Extraction conditions of antithrombin active materials

Experimental groups were divided into five. In each group, 10 g of red ginseng powder was suspended with 50 ml of distilled water and pHs of slurry were respectively adjusted into 3, 5, 7, 9 and 11 by 1 N HCl or 1 N NaOH. The slurries were respectively stirred for 2 hours at room temperature, and then centrifuged at 11,000 g for 20 min. Antithrombin activities of the supernatants were measured, respectively.

4. Isolation of a polysaccharide with antithrombin activity

Red ginseng (20 g-powder) was suspended in 200 ml distilled water and pH of slurry was adjusted into 9.0 by 1 N NaOH. The slurry was stirred for 2 h at room temperature and centrifuged at 11,000 g for 20 min. 200 mM Tris buffer (pH 8.0) was added to the supernatant to make its final concentration to be 50 mM Tris buffer (pH 8.0). The solution was loaded onto a DEAE-Sepharose column (10.0 cm × 2.5 cm) equilibrated with 50 mM Tris buffer (pH 8.0) and washed with the same buffer. The elution was done with a linear gradient of NaCl (0~0.5 M) in 50 mM Tris buffer (pH 8.0) at a flow rate of 60 ml/h. The active fractions with antithrombin activity were pooled and dialyzed against distilled water. The partially purified preparations were applied on a Sepharose CL-6B column (115 cm× 1.28 cm) and eluted with 50 mM Tris buffer (pH 8.0) containing 0.1 M NaCl at a flow rate of 30 ml/h. The active fractions were pooled, dialyzed against distilled water, and lyophilized.

5. Cellulose acetate membrane electrophoresis

Electrophoresis was performed by a method of Lee *et al*[7] on a cellulose acetate membrane. After electrophoretic run in 0.1 N HCl, the membrane was stained with 0.1% Toluidine blue in 3% acetic acid and then destained with 3% acetic acid.

6. Determination of molecular weight

The average molecular weight of isolated polysaccharide was estimated as in Zhuang *et al*[16] using a Sepharose CL-6B gel filtration column equilibrated with 50 mM Tris buffer (pH 8.0) containing 0.1 M NaCl. A calibration curve was obtained with dextrans (MW 464, 282, 148 and 67 kDa) as molecular weight markers.

7. Chemical analyses

Sugar content was determined by the phenol-sulfuric acid method with glucose as the standard, as described by Dubios *et al.*[17] Uronic acid was measured by the modified *m*-hydroxydiphenyl-sulfuric acid method of Blumenkrantz and Asboe-Hansen[18] with D-glucuronic acid as the standard. Sulfate content was determined by the turbidimetric method of Dogson and Price[19] with K_2SO_4 as the standard. Neutral sugars were analyzed as in Jones and Albersheim.[20] Isolated polysaccharide (2 mg) was hydrolyzed with 2.0 M trifluoroacetic acid at 121°C for 6 h, and the alditol acetate derivatives of sugars were mea-

sured by gas chromatography equipped with a Supelco SP-2380 column (Supelco, Bellefonte, PA): myo-inositol was used as an internal standard.

8. Assays for plasma coagulation

The activated partial thromboplastin time (aPTT), prothrombin time (PT) and thrombin time (TT) were measured by the procedures adopted from Fox *et al*[21] using a blood coagulation analyzer. A mixture of polysaccharide solution (15 μl, 0~625 μg/ml), citrated pooled human plasma (100 μl) and aPTT reagent (100 μl) was incubated for 3 min at 37°C, and aPTT was measured after the addition of 100 μl of 20 mM $CaCl_2$ to the mixture. After preincubation of citrated pooled human plasma (100 μl) and polysaccharide solution (15 μl, 0~625 μg/ml) for 3 min at 37°C, PT and TT were measured after the addition of 200 μl of thromboplastin C reagent and the addition of 100 μl of thrombin reagent, respectively.

Results and Discussion

As shown in Fig. 1, water-extraction of antithrombin active materials from red ginseng was ineffective below pH 5.0 but rapidly increased as pH was increased ranging from 5 to 7. Since, above pH 9, extraction of active compounds was nearly constant, the pH for optimum condition of extraction was chosen as 9. Antithrombin active polysaccharide from akaline extract of red ginseng was isolated by a combination of anion exchange chromatography and gel filtration chromatography. The ion exchange chromatography eluted the antithrombin active materials of red ginseng into near 0.3 M NaCl concentration. Further purification was performed with active fractions by Sepharose CL-6B chromatography, which gave rise to a chromatogram with virtually a single and symmetrical band of polysaccharide showing antithrombin activity (Fig. 2).

Cellulose acetate membrane electrophoresis showed that the antithrombin active compound behaved as a sharply single band (Fig. 3). This result indicated that antithrombin active compound was purified nearly to homogeneity as an acidic polysaccharide. The average molecular mass of this polysaccharide was estimated to be about 177 kDa by Sepharose CL-6B gel fil-

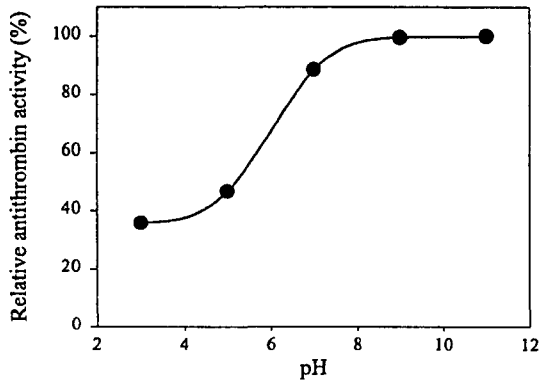

Fig. 1. Effect of pH on extraction of antithrombin active materials in red ginseng.

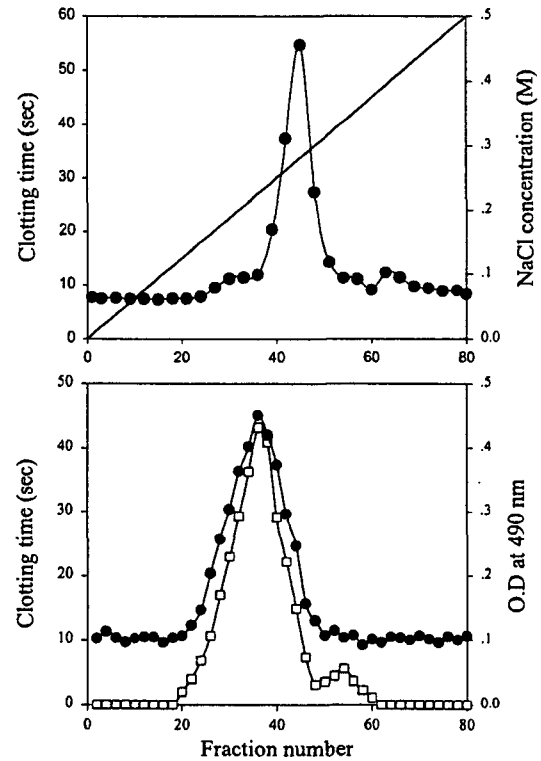

Fig. 2. Isolation of the polysaccharide with antithrombin activity using DEAE-Sepharose ion exchange chromatography (A) and Sepharose CL-6B gel filtration chromatography (B). The antithrombin activity was measured by an increase in fibrin clotting time, and sugar content was determined by the phenol-sulfuric acid method. Closed circles, antithrombin activity; Open rectangles, sugar cotent; solid line, salt linear gradient.

Fig. 3. Electrophoresis of active polysaccharide on a cellulose acetate membranes. Electrophoresis was performed in 0.1 N HCl for 1 hr at 3 mA/cm.

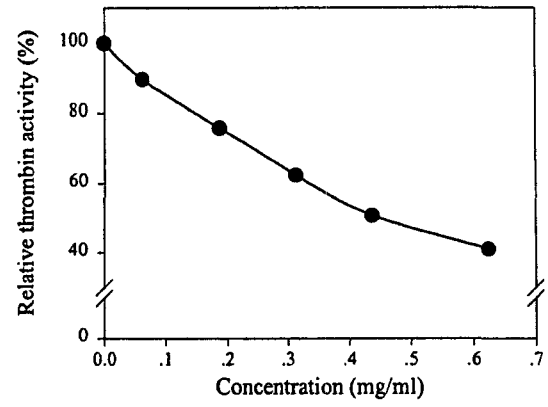

Fig. 4. Effects of active polysaccharide on fibrinogen clotting activity of thrombin. Data are presented as mean (n=3).

tration using dextrans as standards. As summarized in Table 1, this polysaccharide was found to be a sulfated heteropolysaccharide, containing substantial amounts of uronic acid (40.2%), sulfate group (9.2%) and protein (1.5%). Neutral sugar of the active polysaccharide consists of rhamnose, mannose, galactose, arabinose, glucose, fucose and xylose in a molar ratio of 1.00 : 0.88 : 0.86 : 0.78 : 0.70 : 0.33 : 0.22. Although number of sulfated polysaccharides with anticoagulant and/or antithrombin activities have been isolated from seaweeds and a mushroom, molecular weight, sulfate content and sugar composition of active polysaccharide in red ginseng are apparently distinctive from those of other active polysaccharides from various sources.[3-7)]

The effect of active polysaccharide on fibrin formation by thrombin were examined (Fig. 4). In the presence of the active polysaccharide in red ginseng, thrombin-catalyzed fibrinogen clotting was inhibited with increasing amount of the polysaccharide added. It was reported that polysaccharide from brown seaweed has antithrombin activity against fibrin clotting and the inhibitory effect of active polysaccharide on fibrinogen-thrombin interaction has been attributed to the steric hinderence arising from the polysaccharide

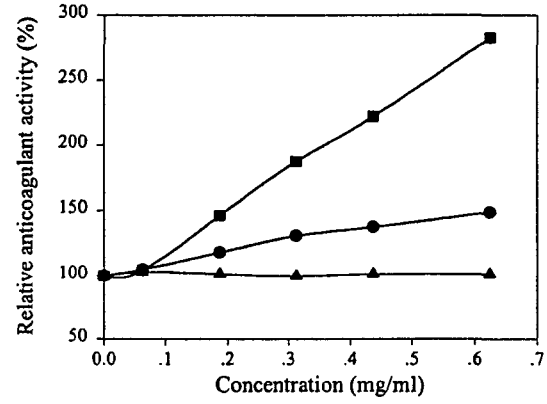

Fig. 5. Effects of active polysaccharide on the process of plasma coagulation. Data for changes in TT (squares), aPTT (circles) and PT (triangles) with increasing concentrations of active polysaccharide in the respective reaction mixtures. Data were presented as the means of triplicated experiments.

binding to fibrinogen.[22-23)] The mechanism of antithrombin effect of active polysaccharide in red ginseng will be investigated. Anticoagulant activity of the polysaccharide was examined with human plasma by use of aPTT, PT and TT assays. As shown in Fig. 5, the anticoagulant action was seen in aPTT and TT in a dose-dependent manner but not in PT, suggesting that the polysaccharide activity may be related to the

Table 1. Chemical compositions of active polysaccharide isolated from red ginseng

Uronic acid (%)	Sulfate (%)	Protein (%)	Neutral sugar composition (molar ratio)						
			Rha.	Man.	Gal.	Ara.	Glc.	Fuc.	Xyl.
40.2	9.2	1.5	1.00	0.88	0.86	0.78	0.70	0.33	0.22

intrinsic coagulation pathway and the third coagulation phase in plasma.[24] The marked increase in TT especially indicated that the third coagulation phase related to the thrombin-mediated fibrin formation was severely inhibited.

In conclusion, we isolated and partially characterized a new polysaccharide with anticoagulant and antithrombin activity in red ginseng. Studies on the effect of the polysaccharide *in vivo* experiment and on its further characterization will be investigated as well as study on the mechanism of antithrombin activity.

요 약

홍삼으로부터 염기성 수용액 추출, 음이온교환 크로마토그래피 및 겔여과 크로마토그래피를 순차적으로 수행하여 항트롬빈 활성을 보이는 다당체를 분리하였다. 이 다당체는 셀룰로스 아세테이트막을 사용한 전기영동에서 하나의 띠로 나타나 비교적 순수하게 정제되었음을 알 수 있었고, 겔여과 크로마토그래피에서 결정된 평균 분자량은 약 177 kDa이었다. 40.2%의 유론산, 9.2%의 황산기 및 1.5%의 단백질을 포함하는 산성다당체였고, 구성 중성당으로 rhamnose, mannose, galactose, arabinose, glucose, fucose, xylose를 1.00 : 0.88 : 0.86 : 0.78 : 0.70 : 0.33 : 0.22의 비율로 포함하고 있었다. 이 다당체는 트롬빈에 의한 피브리노겐의 응고를 농도에 비례하여 저해하였고, 혈장을 사용한 실험에서 내인성 경로를 통해 혈액응고를 저해하는 것으로 나타났다.

Acknowledgements

This work was supported by grants for ginseng from Korea Tobacco and Ginseng Cooperation (1999). We also feel grateful to Korea Tobacco and Ginseng Cooperation for the supply of red ginseng.

References

1. Jackson, C. M. : *Ann. Rev. Biochem.* **49**, 765-811 (1980).
2. Gibaldi, M. and Wittkowsky, A. K. : *J. Clin. Pharmacol.* **35**, 1031-1045 (1995).
3. Nishino, T., Yokoyama, G., Dobashi, K., Fusihara, M., and Nagumo, T. : *Carbohydr. Res.* **186**(1), 119-129 (1989).
4. Dobashi, K., Nishino, T., Fusihara, M., and Nagumo, T. : *Carbohydr. Res.* **194**, 315-320 (1989).
5. Nishino, T., Takabe, Y., and Nagumo, T. : *Carbohydr. Polym.* **23**, 165-173 (1994).
6. Lee, H.-S., Kweon, M.-H., Lim, W.-J., Sung, H.-C., and Yang, H.-C. : *Korean J. Food Sci. Technol.* **29**(2), 369-375 (1997).
7. Lee, H. -S., Jin, S. -H., Kim, H. S., and Ryo, B. -H. : *Korean J. Food Sci. Technol.* **27**(5), 716-723 (1995).
8. Dürig, J., Bruhn, T., Zurborn, K. -H., Gutensohn, K., Bruhn, H. D., and Béress. L. : *Thromb. Res.* **85**(6), 479-491 (1997).
9. Sakada, T., Etou, H., Fujimoto, K., Hayashi, T. and Arichi, S. : *Korean J. Ginseng Sci.* **11**, 164-172 (1987).
10. Matsuda, H., Kubo, M. and Tani, T. : *Shoyakugaku Zasshi* **41**(2), 135-141 (1987).
11. Hwang, W. I. : *Korean J. Ginseng Sci.* **17**, 52-60 (1993).
12. Kim, Y. S., Kang, K. S. and Kim, S. I. : *Arch. Pharm. Res.* **13**(4), 330-337 (1990).
13. Konno, C. and Hikino, H. : *Int. J. Crude Drug Res.* **25**(1), 53-56 (1987).
14. Gao, Q. P., Kiyohra, H., Cyong, J. C. and Yamada, H. : *Planta Medica* **55**, 9-12 (1989).
15. Carpiello, M., Vilardo, P. G., Lippi, A., Criscuoli, M., Corso, A. D., and Mura, U. : *Biochem. Pharmacol.* **52**, 1141-1146 (1996).
16. Zhuang, C., Itoh, H., Mizuno, T. and Ito, H. : *Biosci. Biotech. Biochem.* **59**, 563-567 (1995).
17. Dubios, M. K. A., Hamilton, T. K., Rebers, P. A., and Sonisth, F. : *Anal. Chem.* **28**, 350 358 (1956).
18. Blumenkrantz, N. and Asboe-Hansen, G. : *Anal. Biochem.* **54**(2), 484-489 (1973).
19. Dogson, K. S. and Price, R. G. : *Biochem. J.* **84**, 106-110 (1962).
20. Jones, T. M. and Albersheim, P. : *Plant Physiol.* **49**, 926-936 (1972).
21. Fox, I., Dawson, A., Londs, P., Eisner, J., Findlen, K., Levin, E., Hanson, D., Mant, T., Wagner, J., and Maraganore, J. : *Thromb. Haemost.* **69**(2), 157-163 (1993).
22. Nishino, T., Aizu, Y., and Nagumo, T. : *Thromb. Res.* **62**, 765-773 (1991).
23. Nishino, T., Aizu, Y., and Nagumo, T. : *Thromb. Res.* **64**, 723-731 (1991).
24. Nishino, T. and Nagumo, T. : *Carbohydr. Res.* **229**, 355-362 (1992).