Thesis for the Degree of Master of Engineering

In Vitro Anticoagulant Activity of Chitosan Sulfate

By

Young-Dong Jeong

Department of Biomedical Engineering

The Graduate School

Pukyong National University

February 26, 2018

In vitro anticoagulant activity of chitosan sulfate

황산화 키토산의 체외 항응고 효과

Advisor: Prof. Won-Kyo Jung

by

Young-Dong Jeong

A thesis submitted in partial fulfillment of the requirement

for the degree of

Master of Engineering

in Interdisciplinary Program of Biomedical Engineering

The Graduate School

Pukyong National University

February 2018

In vitro Anticoagulant Activity of Chitosan Sulfate

A dissertation

by

Young-dong Jeong

Approved by:

(Chairman) Young-Mog Kim Ph. D

(Member) Seong-Yun Nam Ph. D

(Member) Won-Kyo Jung Ph. D

February 26, 2018

In vitro anticoagulant activity of chitosan sulfate

Young-dong Jeong

Department of Biomedical Engineering,

Center for Marine-integrated Biomedical Technology (BK21 plus)

The Graduate School

Pukyoung National University

Abstract

키토산(chitosan)은 게, 새우등 갑각류와 버섯과 같은 균류의 단백질 복합체 로써, 생물체의 외피와 골격을 구성하고있는 키틴(chitin)을 탈아세틸화 하여 얻어지는 다수의 아미노기를 가지고있는 다당이다. 셀룰로오스와 유사한 구조를 가지고 있고, 셀룰로오스의 C-2위치의 수산기(-OH)가 아민기($-NH_2$)로 치환된 형태이다. 그러나 키틴은 아세틸아미노기가 수소결합을하여 강한 구조를 형성하기 때문에 유기용매에 용해성이 좋지 않다. 키토산은 희석한 산용액에 잘 용해되기 때문에 다양하게 산업적으로 이용이 가능하다.

혈액에는 여러가지 응고인자가 많이 있어 응고인자가 활성화 되고, 응고기전에 의해 상처가 생기게 되면 지혈이 일어나게 된다. 혈액의 항응고제는 응고기전 중 한단계를 차단하여 활성화 되지 못하게 하여 혈액이 응고되지 못하게 한다. 항응고제는 혈전이 생길위험이 높은 경색증 환자에게 투여하여 응고를 억제하고, 혈액 검사시 응고가 되지 않는 검사에 사용된다. 항응고제의 종류로는 EDTA, Heparin, sodium citrate, sodium oxalate 등이 있다. EDTA, sodium citrate, sodium oxalate는 혈액중의 칼슘이온과 결합하여 응고기전이 진행되지 못하게 하여 항응고 효과를 나타내고, 헤파린은 antithrombinIII 나 heparin cofactorII의 활성을 증가시켜 강력한 항응고 효과를 나타낸다. 현제 헤파린은 항응고제로서 가장 많이 사용되고 있다. 하지만 가격이 비싸다는 단점이 있다. 따라서 천연 다당류를 이용하여 헤파린과 비슷한 항응고제를 개발하는 연구가 이루어져왔다.

본 연구는 키토산을 황산화 시켜 분자량과, 분자비에 따라 나누어 N – hexanoyl chitosan sulfate(HSCOS)를 분리하여 APTT, PT, TT 를 측정하고 헤파린과 비교하여 응고 억제 효과가 얼마나 있는지 확인하였다. 헤파린 만큼의 억제 효과를 가지지는 않지만 APTT와 TT를 연장 시키는 것을 보아 내인계 응고인자와 공통계에 영향을 주는 것을 알 수 있었다. 표면 플라스몬 공명(surface plasmon resonance, SPR)을 통해 어떤 응고 인자를 저해하는지 보니 2 번인자와 10 번인자 둘다 저해를 하지만, 10 번인자를 2 배 정도 강하게 저해 효과를 나타내는 것을 볼 수 있었다. 황산화 키토산은 추후 연구를 통해 항응고재로 사용할 수 있을 것으로 사료된다.

Table of contents

List of table

List of figure

1. Introduction

1.1 Hemostasis / Coagulant pathway

The hemostatic system maintains blood in a normal conditions state [1]. If blood vessels damaged, hemostasis begins. When blood vessels are destroyed, collagen in the vessel wall appears. von willebrand facter (vWF) in subendothelium binding GPIb/IX complex of platelets then binding collagen in the vessel wall. The attached platelets secrete adenosine diphosphate (ADP), serotonin, cacium ion, vWF and fibrinonectin. Secreted ADP binding surrounding platelet. In combination with platelet, platelets are activated, resulting in GPIIb/IIIa change structure in platelets. Platelet aggregation occurs through the fibrinogen source and a platelet plug is formed [1] (fig 2).

The blood coagulation pathway involves many coagulation factors. When the human body gets injury, a hemostasis occurs. The human blood coagulation cascade is composed of three pathway (intrinsic pathway, extrinsic pathway, common pathway). This pathway leads to fibrin formation. Both intrinsic pathway (contact activation), and extrinsic pathway (tissue factor pathway) are same purpose that produces fibrin. The intrinsic and extrinsic pathway both activate the common pathway. The common pathway is to produce fibrin by activated factorX. And many various substances are required for coagulation cascade such as calcium, phospholipid vitamin K [2].

Intrinsic pathway begins with formation of the collagen by high-molecular-weight kininogen (HMWK), prekallikrein, and Factor XII. Activated Factor XII converts Factor XI into Factor XIa. Factor XIa activates Factor IX. Activated Factor IX and factor VIII activate Factor X together. When factor X is activated, common pathway become active [3].

Extrinsic pathway starts damage to blood vessel, factor VII contact with tissue factor, forming an activated complex. TF-VIIa activates factor X [4].

Common pathway is activated factor X by TF-VIIa and IXa turns prothrombin into thrombin. Thrombin makes fibrinogen into fibrin. Soluble fibrin monomer turn into stable fibrin polymer by means of activated factor XIII (Fig. 1) (Table. 1).

1.2 Natural anticoagulants

Coagulation system is a complex of anticoagulant proteins, a suitable balance ensures fibrinolysis and hemostasis in human body [5].

Tissue factor pathway inhibitor (TFPI)

Extrinsic pathway inhibitor. TFPI forms a complex of activated Factor X to inhibit the activity. And FXa/TFPI complex increase inhibiting ability of FXa/TFPI attached to the cell membrane.

Protein C

Protein C is vitamin-K-dependent plasma protein. Thrombin-thrombomodulin complex activate protein C. activated protein C forms a complex of protein S, the complex contributes to inactivation of factor Va and factor VIIIa.

Protein S

Protein S is cofactor. Protein S either bound to C4b-binding protein or feely in plasma. But only free protein S is activate for protein C[6]

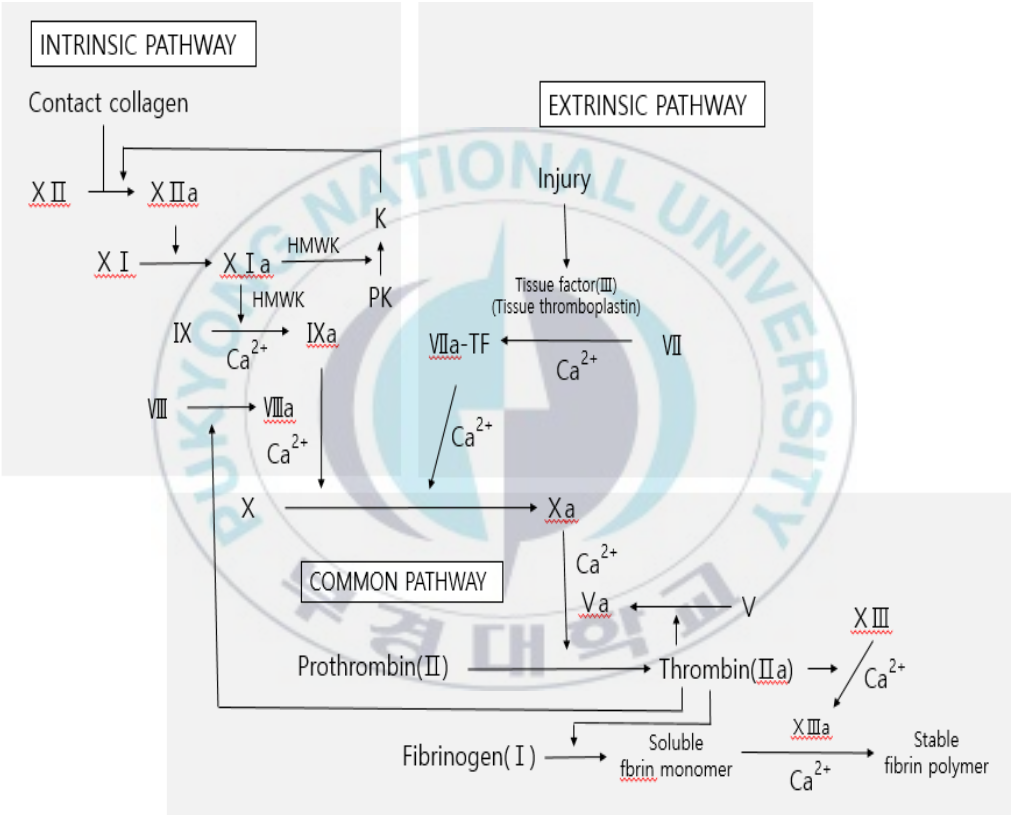


Figure 1. Coagulant pathway

Symbol	Synonym	Mean plasma conc.	Molecula weight(Da)
I	Fibrionogen	200~400 mg/dL	340×10^3
II	Prothrombin	15~15 mg/dL	68×10^3
III	Tissue thromboplastin	None	44×10^3
IV	Calcium ion	8~10 mg/dL	
V	Proaccelerin	1 mg/dL	480×10^3
VII	roconvertin	0.05 mg/dL	59×10^3
VIII	Antihemophilic factor A	0.01 mg/dL	$1\sim2 \times 10^3$
IX	Antihemophilic factor B	0.3 mg/dL	55×10^3
X	Stout prower factor	1 mg/dL	63×10^3
XI	Plasma thromboplastin antecedent	0.5 mg/dL	160×10^3
XII	Hegeman factor	3 mg/dL	82×10^3
XIII	Fibrin stabilizing factor	2 mg/dL	35×10^4
-	High molecular weight kininogen	5 mg/dL	11×10^4
-	prekallikrein	35~50μg/mL	80×10^3

Table 1. Coagulant factor

(http://www.thrombocyte.com/clotting-factors)

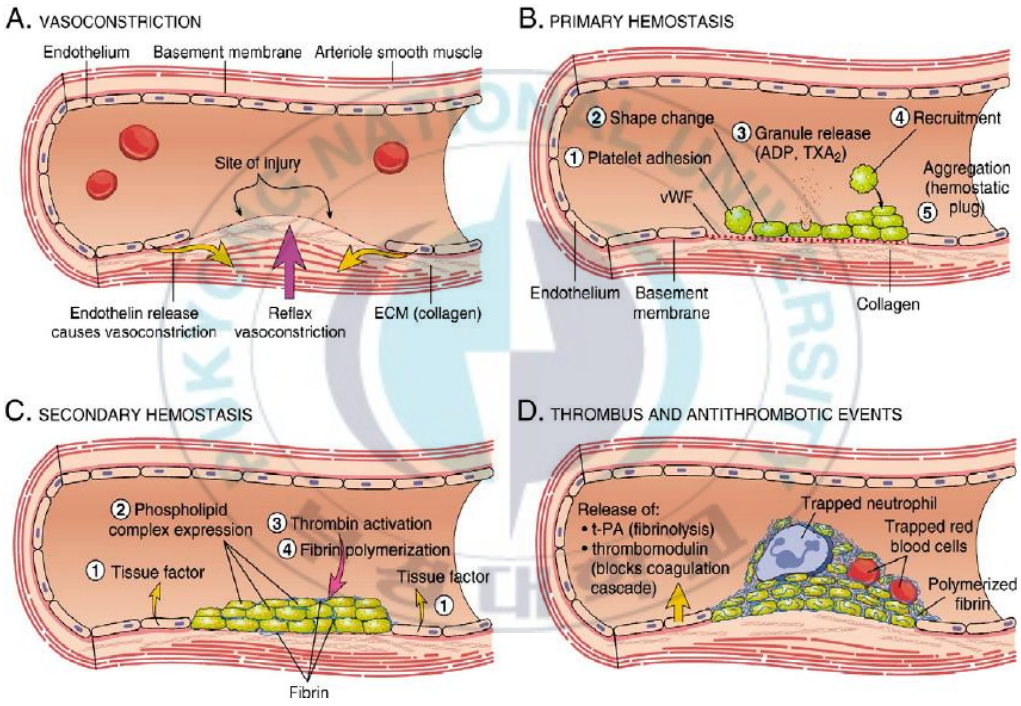


Figure 2. Blood coagulant system

(http://www.writeopinions.com/hemostasis)

Antithrombin III (heparin cofactor I)

Antithrombin III mainly thrombin inhibitor. It is also effect on coagulation factor Xa, factor IXa, factor XII, factor XI and kallikrein. ATIII accounts for 70~80% of inhibitory mechanisms. The presence of heparin significantly increases the rate of inhibition.

(Table 2.)

1.3 Chitin / Chitosan / Sulfated chitosan

Chitin is a naturally occurring biopolymer found in various living organisms such as crustacean, insects, and fungi, where it is a major constituent of the exoskeleton, and it is the second abundant polysaccharide which composed poly (β-(1-4)-N-acetyl -D-glucosamine) in the world [7] (Figure 4A). However, it has unique applications of the biological properties due to water insolubility value while it is environmentally friendly, biocompatible, and biodegradable biopolymer [8] .

Chitosan is a cationic heteropolysaccharide that the partially or fully deacetylated derivative of chitin under alkaline conditions or enzymatic hydrolysis. The structure of chitosan was composed of β-1,4,-linked glucosamine units, D-glucosamine and N-acetyl- D-glucosamine [9] (Figure 4B). It has been reported biological activities such as anti-microbial[10], anti-tumor , wound healing [11], anti-coagulant [12], reactive oxygen species (ROS) scavenging [13] and immune enhancing properties [14] as well as nontoxicity, biodegradability, and biocompatibility [8]. Compared with chitin, chitosan exhibits highly solubility in organic acid and application in the food, pharmaceutical and biomedical industries [15]. Although chitosan has been reported biological activities in the widely field, it has still limited food and medicine application due to poor solubility of high molecular weight (MW) in aqueous media.

Inhibitor	Plasma Concentration(μg/mL)	MW	Major target enzymes
$\boldsymbol{a_1}$ protease inhibitor	2,500	55,000	F-XI, elastase
Antithorombin III	290	62,000	F-Xa, thrombin
$\boldsymbol{a_2}$ Macroglobulin	2,500	725,000	Kalikrein, plasmin, thrombin
C1 inhibitor	240	105,000	F-XIIa, kallikrein
$\boldsymbol{a_2}$ antiplasmin	70	67,000	Plasmin
Heparin cofactorII	40	65,000	Thrombin
PAI-1	10	50,000	Plasmin
Protein C inhibitor(PAI-3)	5	53,000	Protein C, kallikrein
Tissue factor pathway inhibitor	0.1	40,000	F-VIIa, F-Xa

Table 2. coagulation inhibitor protein in plasma

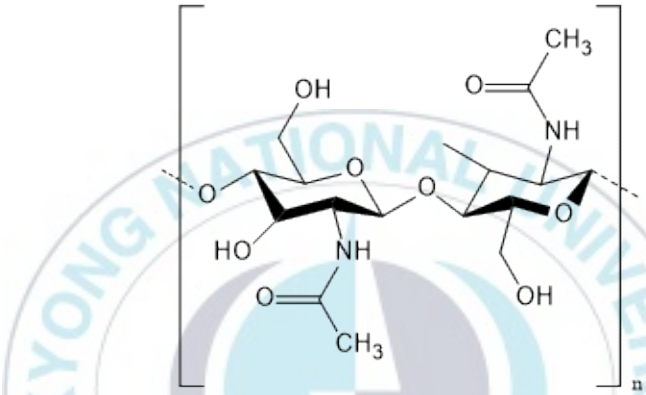
Chitin and chitosan are recommended natural polymer because it has good property, such as non-toxicity, biodegradability, biocompatibility and adsorption properties [16]. But these polyssacarides limitaion in their reactivity and processability.

Sulfate chitosan natural semisynthetic. Those are various biological activities., Such as antioxidant, antiviral, anticoagulant.[17] One of attractive activities is anticoagulant [12]. Some cases increasing the degree of sulfation was grow in anticoagulant activity. Thrombin time(TT) seem to be involve in anticoagulant. Sulfated chitosan interesting bipolymer and similar effect to heparin [18].

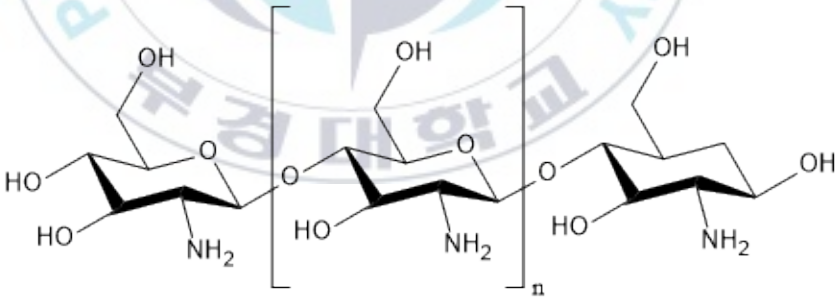
1.4 Surface plasmon resonance (SPR) analysis

Surface plasmon resonance identify to blood coagulation factors, with binding affinity. SPR is the resonant oscillation of conduction electrons at between positive and negative material measure by light. Measuring absorption of material on to metal surface or onto the metal nanopaticles [19]. SPR occurs when polarized light strikes an eletrically conducting surface at the interface between two media. When molecule is bonded to a ligand on a metal surface, light refraction is induced and show on the graph (fig 4.).

(A)



(B)



R= H or Ac, n= 0 to 8

figure 3. (A) Chitin (B) Chitosan

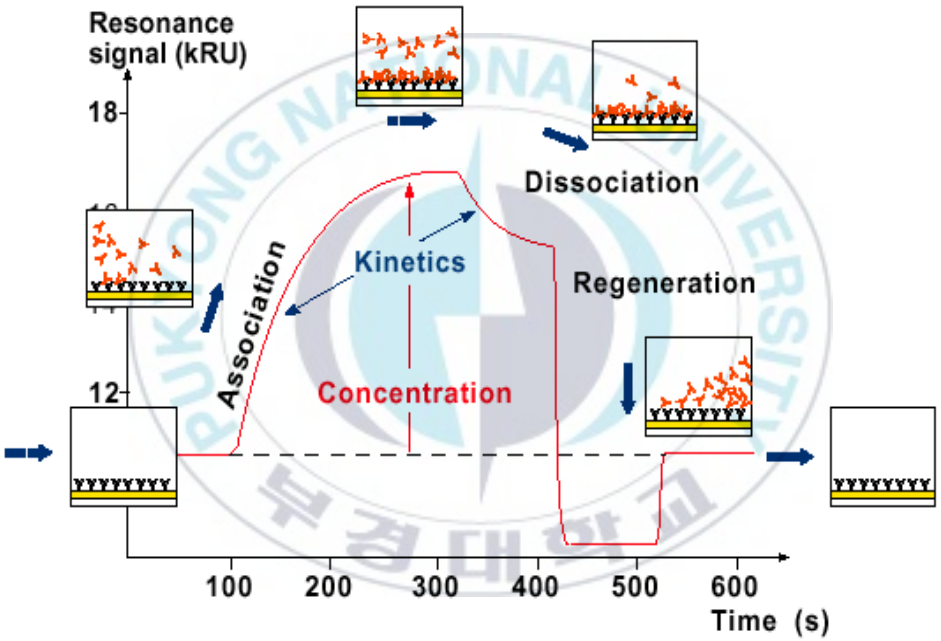


Figure 4. Surface plasmon resonance(SPR). First ligand is attached to the sensor chip and only buffer only flows, no change graph. When sample is injected, it binds with ligand and increase RU. No change when all ligand and sample react. When buffer is reinserted, sample bound to ligand is dropped and RU slightly lowered. Finally, the step of regenerating sensor chip is to wash bound sample by flowing low HCL. (https://www.creativebiomart.net)

2. Materials and methods

2.1 Materials

Chitosan was purchased from Chitolife (Seoul, South Korea). An ACL® Coagulation analyzer (ACL® 7000), a blood coagulation assay kit (HemosILTM APTT Lyophilized silica for APTT, HemosILTM TT, and HemosILTM), and a Factors-Deficient Plasma were produced by the Instrumentation Laboratory Co. (Lexington, MA, USA) Human blood coagulant factors (zymogens of FII, FV, FVII, FIX, FX, FXI, FXII, and their serine proteinase type enzymes of the activated FII, FV, FVII, FIX, FX, FXI, FXII) and 6-amino-1-naphthalenesulphonamide (ANSN)-based fluorogenic substrates (SN-7; Mes-D-LGR-ANSN$(C_2H_5)_2$ and SN-59; D-VPR-ANSNHC_4H_9 2HCl) were obtained from Haematologic Technologies, Inc. (Essex Junction, VT, USA). Fibrinogen, plasmin, and molecular markers for electrophoresis were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The BIAcore® 2000 system for surface plasmon resonance (SPR) analysis, a sensor chip CM5, surfactant P20, HBS buffer (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, 0.05% surfactant P20, pH 7.4), an amine-coupling kit containing N-hydroxysuccinimide (NHS), N-ethyl-N0-(3-diethylaminopropyl) carbodiimide (EDS) and ethanolamine hydrochloride were obtained from Biacore AB Co. (Uppsala, Sweden).

2.2 Chitosan sulfate

Chitosan is added to the sulfating reagent consisting of $CISO_3H$ (100 ml) and formamide (150 ml). mixture is stirred at 80~90℃ for 4 h. and C_3H_6O (500 ml) was added to the precipitate product. Precipitate dissolve in distilled water. And control the pH to 10-11. The mixture is dialyzed for 24 h and concentrated. Chitosan sulfate was

dissolved in methanol (15 ml) and 10% acetic acid (10 ml), and stirring. After 1 h add propanoic anhydride at room temperature, and divide molar ratio. Adjusted to pH 9.0-10.0 and Stirring for 4 h. And added 1M NaOH. After then dialyze for 24 h (Table 3.). This experiment used sulfated chitosan with a molecular ratio 2.5:2. This is N-hecanoyl chitosan sulfate(HSCOS)

2.3 Investigation of anticoagulant activity

Activated partial thromboplastin time tests for intrinsic pathway and common pathway. APTT measure the time until fibrin is clot, after calcium and an activating agent add to plasma. First blood is drawn into a test tube containing oxalate or citrate. The blood mixed, then centrifuged to separate plasma. Normal citrated plasma (80 μl) added anticoagulant solution (20 μl). PT reagent warmed (200 μl) is added to mixture plasma and the time is measured. Prothrombin time (PT) test for extrinsic pathway and common pathway. APTT measure the time until fibrin clot, after add to optimal amount of calcium and an excess of thromboplastin are added to decalcified plasma [20]. For activated partial thromboplastin time (APTT), reagent (100 μl) is added to plasma (100 μl) warmed for 3min, $CaCl_2$ (100 μl, 20 mM) is added and clotting time recorded. Thrombin time (TT) is fibrin clotting time after adding thrombin (100 μl, 3min at 37°C) to mixture plasma (200 μl).

2.4 Surface plasmon resonance(SPR)

Surface plasmon resonance (SPR) is optical method for measuring material. This method measure up refractive index of thin layers of metal, has target material [19]. Confirm the blood coagulant factor with surface plasmon resonance (SPR) using BIAcore® 2000. The coagulation factor in common pathway and intrinsic pathway are fixed CM5 chip of the dextran surface. The analyte inject the sensor chip, the

coagulation factors in the buffer are coupled to the sensor chip. binding kinetics of analytes was measured (*ka, kd,* K_D). Association rate constant (*ka*) is calculate multiple sensorgrams, different concentrations of each experiment. Dissociation rate constant (*kd*) is calculated dissociation phase of binding curve. Equilibrium dissociation constant (K_D) is the ratio of *ka/kd* using BIAevaluation software.

sample	Molar ratio	Elemental analysis(wt%)				degree of substitution	$M_v(\times 10^3 g\ mol^{-1})$
		S%	C%	N%	H%		
1		10.90	32.20	4.50	5.93		6.87
2	0.5:2	10.15	33.63	5.54	4.90	0.18	5.89
3	1.5:2	8.95	38.40	4.88	5.52	0.53	5.35
4	2.5:2	8.30	40.83	4.54	5.85	0.75	6.57

Table 3. Sulfated chitosan. Sample number 4 is N – hexanoyl chitosan sulfate (HSCOS)

3. Results

3.1. In vitro coagulation assay (in plasma)

APTT, PT, and TT, to know what HSCOS effecting the coagulation process. The anticoagulant activity measured by prothrombin time, thrombin time, activated partial thromboplastin time. The results according to concentration can be seen fig 5. When using heparin, result is 240sec at a concentration 5 μg/ml[21]. When the HSCOS concentration was 25 μg/ml, APTT was 110 sec, and when concentration was 100 μg/ml, APTT is 182 sec. TT was 25 μg/ml at 60.7 sec, and 100 μg/ml at 80.9 sec. PT was 25 μg/ml at 11.3 sec, 100 μg/ml at 11.9 sec. The results show that activated partial thromboplastin time (APTT) potently prolonge as the concentration increases. Thrombin time (TT) also slightly increased as the concentration increases. But prothrombin time (PT) was not prorlonged by HSCOS. There results suggested that HSCOS could inhibit specific intrinsic and common pathway factor.

The concentration of HSCOS increases, the coagulation factor is inhibited and residual activity is increase. These results show that as HCOS concentration increase, Fxase and prothrombinase increase. There is no difference in effect at 50 μg/ml (Fig 6.).

3.2. Binding affinity assay

Results in the blood coagulation assay, HSCOS uses surface plasmon resonance (SPR) to determine intrinsic and common pathway factors affect. The dissociation (k_d) association rate constant (k_a) and equililbrium dissociation constant ($k_D = k_d / k_a$) ware Table 4. HSCOS effects with ATIII in human coagulation factor and affect the common pathway factor.

There is a difference in the activity of inhibiting the coagulation factor with antithrombin III and without antithrombin III (table 5.). When antithrombin III is

present, coagulation factor Xa, factor IIa inhibitory activity were high. On the other hand coagulation factor inhibition is low when absence antithrombin III.

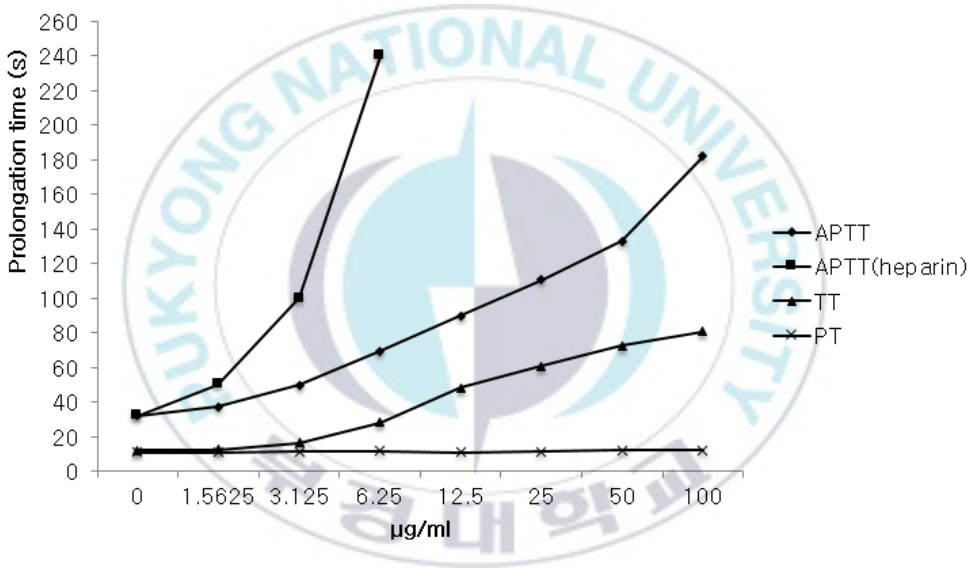


Figure 5. Result of APTT, APTT(heparin), PT and TT according to concentration of N-hexanoyl chitosan sulfate (HSCOS)

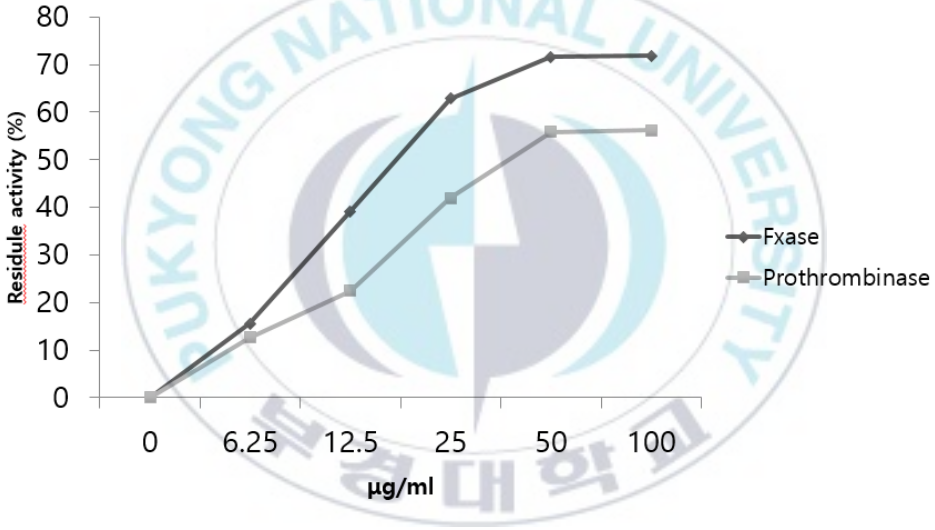


Figure 6. Effect of HSCOS on residual activity of plate poor plasma (PPP), Fxase and prothrombinase

Analyte	Ligand	Association rate constant (ka) M−1s−1	Dissociation rate constant (kd) M−1s−1	Equilibrium dissociation constant (KD=kd/ka) nM
HSCOS *(Present ATIII)*	FXa	0.96×10^{3}	7.02×10^{-3}	67.4
	FIIa	0.28×10^{3}	4.02×10^{-2}	112.6

Table 4. kinetic parameters for the blood coagulation factors with an enzymatic hydrolysate of HSCOS using surface plasmon resonance (SPR) sensorgraphy.

	ATⅢ +	ATⅢ -
Factor		
FXa	68.4 %	10.4 %
FⅡa	37.1 %	5.4 %

Table 5. coagulation factor Xa, factor IIa inhibitory activity in presence or absence antithrombin*III*

4. Discussion

Bioactive compounds can have a positive and negative effect on the blood coagulation system. Natural anticoagulants are medically beneficial, treatment of blood related disorders. In this study, N-hexanoyl chitosan sulfate (HSCOS) prolonged the activated partial thromboplastin time (APTT) and thrombin time (TT) as concentration increases. However, prothrombin time (PT) did not change with increasing concentration. As a result, HSCOS influence intrinsic pathway and common pathway.

Effect of HSCOS on residual activity of plate poor plasma (PPP), as the concentration increases, the residual activity increase. And no difference at more than 50 μg/ml.

In surface plasmon resonance (SPR), HSCOS and antithrombin III act on the common pathway factor II and X. And when antithrombin III is present in activated factor X and II, inhibitory activity is high than when there is no antithrombin III. So HSCOS interacts with antithrombin III, influence activated factor II and X. HSCOS works similar reaction to heparin. Heparin affect intrinsic coagulation factor and common pathway factor with antithrombin III. HSCOS and antithrombin III together inhibit activated factor II, IX, X, XI, XII. HSCOS inhibits factor 10 about twice as strongly as factor 2.

In the study, we have been investigated anticoagulant effects of N-hexanoyl chitosan sulfate (HSCOS) on the blood coagulation pathway. This feature can be used to prevent thrombosis in blood vessels. And the characteristics of N-hexanoyl chitosan sulfate (HSCOS) are considered to be useful for pharmaceutical and nutraceutical and medical treatment.

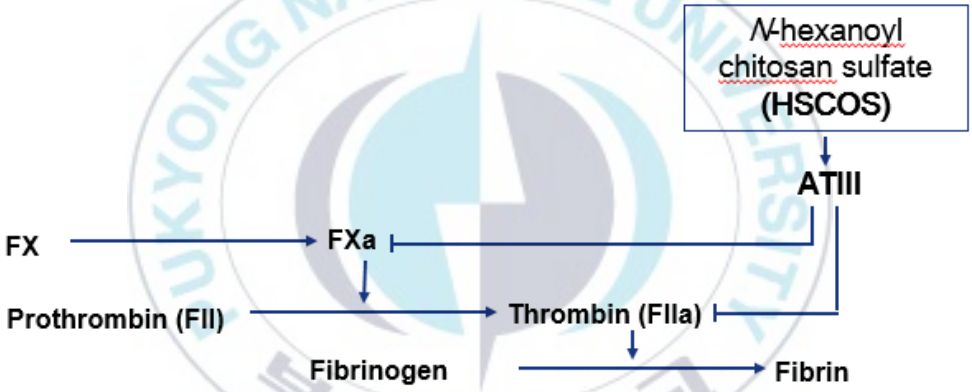


Figure 7. Anticoagulant effect of HSCOS in blood coagulation cascade. HSCOS and antithrombin III have the effect of inhibiting serine protease.

5. References

[1] Mackman N. Role of tissue factor in hemostasis, thrombosis, and vascular development. Arteriosclerosis, thrombosis, and vascular biology. 2004;24:1015-22.

[2] Jung W-K, Kim S-K. Isolation and characterisation of an anticoagulant oligopeptide from blue mussel, Mytilus edulis. Food Chemistry. 2009;117:687-92.

[3] Mosnier LO, von dem Borne PAK, Meijers JC, Bouma BN. Plasma TAFI levels influence the clot lysis time in healthy individuals in the presence of an intact intrinsic pathway of coagulation. Thrombosis and haemostasis. 1998;80:829-35.

[4] Rao L, Rapaport SI. Studies of a mechanism inhibiting the initiation of the extrinsic pathway of coagulation. Blood. 1987;69:645-51.

[5] Hoffman M. Remodeling the blood coagulation cascade. Journal of thrombosis and thrombolysis. 2003;16:17-20.

[6] Larsen TB, Nielsen JN, Fredholm L, Lund ED, Brandslund I, Munkholm P, et al. Platelets and anticoagulant capacity in patients with inflammatory bowel disease. Pathophysiology of haemostasis and thrombosis. 2002;32:92-6.

[7] Jayakumar R, Menon D, Manzoor K, Nair S, Tamura H. Biomedical applications of chitin and chitosan based nanomaterials—A short review. Carbohydrate Polymers. 2010;82:227-32.

[8] Zargar V, Asghari M, Dashti A. A review on chitin and chitosan polymers: structure, chemistry, solubility, derivatives, and applications. ChemBioEng Reviews. 2015;2:204-26.

[9] Narayanan D, Jayakumar R, Chennazhi K. Versatile carboxymethyl chitin and chitosan nanomaterials: a review. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology. 2014;6:574-98.

[10] Xing K, Zhu X, Peng X, Qin S. Chitosan antimicrobial and eliciting properties for pest control in agriculture: a review. Agronomy for Sustainable Development. 2015;35:569-88.

[11] Chandika P, Ko S-C, Jung W-K. Marine-derived biological macromolecule-based biomaterials for wound healing and skin tissue regeneration. International journal of biological macromolecules. 2015;77:24-35.

[12] Huang R, Du Y, Yang J, Fan L. Influence of functional groups on the in vitro anticoagulant activity of chitosan sulfate. Carbohydrate Research. 2003;338:483-9.

[13] Je J-Y, Kim S-K. Reactive oxygen species scavenging activity of aminoderivatized chitosan with different degree of deacetylation. Bioorganic & medicinal chemistry. 2006;14:5989-94.

[14] Zaharoff DA, Rogers CJ, Hance KW, Schlom J, Greiner JW. Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. Vaccine. 2007;25:2085-94.

[15] Fang X, Xu Y, Zhang J, Lu X, Wang Y, Chen M. Synthesis and characterization of an amphiphilic linoleic acid-g-quaternary chitosan with low toxicity. Journal of Nanomaterials. 2015;16:281.

[16] Vongchan P, Sajomsang W, Subyen D, Kongtawelert P. Anticoagulant activity of a sulfated chitosan. Carbohydrate research. 2002;337:1239-42.

[17] Seedevi P, Moovendhan M, Vairamani S, Shanmugam A. Evaluation of antioxidant activities and chemical analysis of sulfated chitosan from Sepia prashadi. International Journal of Biological Macromolecules. 2017;99:519-29.

[18] Campelo CS, Lima LD, Rebêlo LM, Mantovani D, Beppu MM, Vieira RS. In vitro evaluation of anti-calcification and anti-coagulation on sulfonated chitosan and carrageenan surfaces. Materials Science and Engineering: C. 2016;59:241-8.

[19] Pattnaik P. Surface plasmon resonance. Applied biochemistry and biotechnology. 2005;126:79-92.

[20] Suchman AL, Griner PF. Diagnostic DecisionDiagnostic Uses of the Activated Partial Thromboplastin Time and Prothrombin Time. Annals of internal medicine. 1986;104:810-6.

[21] Brito AS, Cavalcante RS, Palhares LC, Hughes AJ, Andrade GP, Yates EA, et al. A non-hemorrhagic hybrid heparin/heparan sulfate with anticoagulant potential. Carbohydrate polymers. 2014;99:372-8.