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Note

Inhibition of the hydrolytic activity of thrombin by chitin heparinoids

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Heparin, an anticoagulant polysaccharide, inhibits the thrombin activity that plays the most important role in blood clotting[1]. Inhibition of thrombin activity is accelerated[2] in the presence of antithrombin III (AT-III). The heparin–AT-III complex also inhibits Factor Xa, which transforms prothrombin into thrombin[3].

As chitin has a similar skeletal structure to heparin, the biomedical properties of chitin have received considerable attention. Chitosan (*N*-deacetylated chitin), has been used to prepare heparin-like derivatives, because it can be chemically modified under homogeneous conditions[4,5] in few reaction steps. Although there have been many attempts[6,7] prepare chitosan heparinoids, the role of each functional group has not been clarified satisfactorily. Use of chitin as a precursor for heparinoids allows the role of ether-linked functional groups to be demonstrated independently of influence by *N*-sulfate groups. Carboxymethylation of chitin was employed, as carboxymethyl groups have been reported[8] to augment the anticoagulant activity of sulfated dextran. Carboxymethylation of chitin to d.s. 0.8 under mild conditions[9] causes mainly 6-*O*-substitution. *O*-(Carboxymethyl)-chitin (CM-chitin) has been reported[10] to adsorb bovine blood proteins, with a positive contribution by Ca^{2+}, and the extent of adsorption is regulated by the degree of substitution.

The present report describes the preparation of various chitin derivatives by sulfation of chitin and CM-chitin under mild conditions, and studies of inhibition of thrombin activity, using bovine fibrinogen. Inhibition was observed to be increased upon introduction of carboxymethyl groups in the sulfated chitin, as also observed for sulfated dextran. The sulfated CM-chitin showed activity comparable to that of

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TABLE I

ANALYSES OF HEPARINOIDS DERIVED FROM CHITIN

Sample	*S (%)*	*SO_3Na*[a]	*CO_2Na*[a]	$[\alpha]_D$[b]	$[\eta]$[c]	*Mol. wt.*[d]
Sulfated chitin						
S-chitin I	4.91	0.48	0	−11.6	0.10	8×10^3
S-chitin II	8.54	0.73	0	−18.6	0.19	1.3×10^4
S-chitin III	10.22	0.87	0	−25.2	0.34	2.4×10^4
Sulfated CM-chitin						
SCM-chitin I	1.43	0.12	0.80	−6.2	1.45	1.8×10^4
SCM-chitin II	6.70	0.57	0.40	−14.6	0.15	1.0×10^4
SCM-chitin III	7.66	0.65	0.56	−21.6	0.34	2.4×10^4
CM-chitin [*O*-(carboxymethyl)chitin]	0	0	0.80	−19.4	5.0	6.3×10^4
HE-chitin [*O*-(hydroxyethyl)chitin]	0	0	0.6[e]	−23.8	1.1	$2\text{–}6 \times 10^4$
P-chitin (chitin phosphate)	0	0	0.8[f]	−11.4	0.15	

[a]Number of functional groups/GlcNAc residues. [b]$C = 0.5$ g/dL in H_2O. [c]The intrinsic viscosities of polysaccharides were measured as a function of the concentration of polymers in 0.1M NaCl by using an Ubbelohde-type viscometer. [d]Average molecular weights (Mv) were determined by using viscosity equations for heparin: $[\eta] = 1.75 \times 10^{-5}\ Mv^{0.98}$ (0.1M NaCl, 25°) as reported by S.E. Lasker *et al.*[14] and for CM-chitin: $[\eta] = 7.92 \times 10^{-5}\ Mv^{1.0}$ (0.1M NaCl, 30°) as reported by M. Kaneko *et al.*[15]. [e]Degree of hydroxyethylation. [f]Degree of phosphorylation.

heparin, even at low concentration in the absence of AT-III. As there was little inhibition by the chitin heparinoids in the presence of AT-III, especially at low concentration, it appears that *N*-sulfate groups, as well as *O*-sulfate and *O*-CM groups are required for full inhibition.

Preparation of sulfated derivatives. — Chitin and CM-chitins were sulfated by the general method of Horton and Just[6]. Chemical analyses of the products are summarized in Table I. The degree of sulfation of chitin was dependent on the reaction time in chlorosulfonic acid–pyridine. The sulfated chitins are denoted as S-chitin I (d.s. 0.48), II (d.s. 0.73), and III (d.s. 0.87). The sulfated CM-chitins (SCM-chitins) were prepared similarly.

The i.r. absorption spectra of the sulfated products showed new peaks at 1240 (S=O stretching) and 820 cm^{-1} (C–O–S stretching). With SCM-chitin III, absorptions at 1240 and 820 cm^{-1} similar to those of S-chitins, are observed. The absorption at 1735 cm^{-1}, attributed to C=O stretching of the carboxyl group, was also confirmed in the protonated sample.

Preliminary ^{13}C-n.m.r. spectral data for S-chitin I and SCM-chitin III showed a downfield shift of only the C-6 signal. There was no significant change in the chemical shift of C-3. It was concluded that O-6 of the GlcNAc residues was the main point of attack under the conditions used. As the chemical shifts of the carbon atoms in the sugar residue were almost unchanged, no configurational change of the chitin molecule nor interaction between the functional groups introduced appeared to have taken place in the solution.

Anticoagulant activity of heparinoids. — The inhibitory action of S-chitins on

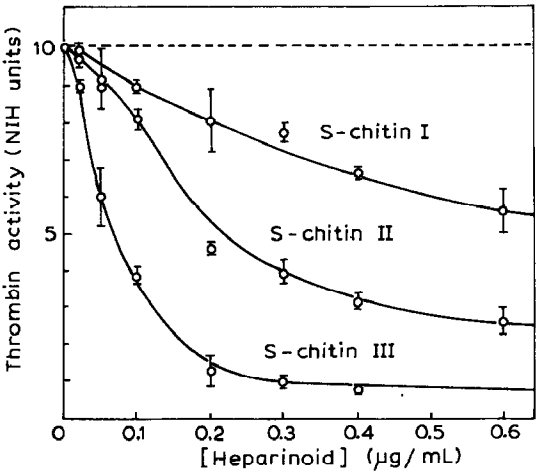

Fig. 1. Inhibitory action of S-chitins (—○—), HE-chitin, CM-chitin, and P-chitin (----) on the transformation of fibrinogen into fibrin by thrombin at pH 7.4 and room temperature. (5mM Tris–HCl buffer containing 0.1M KCl and 5mM $CaCl_2$). Thrombin activity (NIH units) was calculated by means of a standard curve.

the anticoagulant activity toward bovine fibrinogen was studied in relation to the degree of sulfation, at pH 7.4 and room temperature (Fig. 1). Increase of the sulfur content caused an increase in the inhibitory action toward thrombin. The inhibitory action by S-chitin was increased considerably by the introduction of carboxyl group into the S-chitin molecule (Fig. 2). The highest degree of inhibition was given by SCM-chitin III, in which the levels of sulfation and carboxylation were equal; and the inhibition was comparable to that of heparin under our experimental

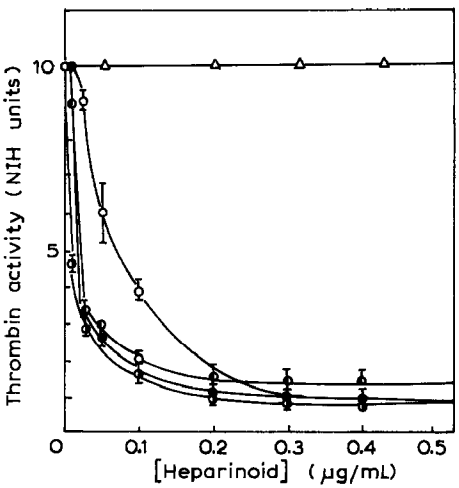

Fig. 2. Inhibitory action of heparin and chitin heparinoids on the transformation of fibrinogen into fibrin at pH 7.4 and room temperature. —●—; Heparin, —△—; SCM-chitin I, —◐—; SCM-chitin II, —◑—; SCM-chitin III and —○—; S-chitin III.

TABLE II

APPARENT BINDING-CONSTANTS AND ANTITHROMBOGENIC ACTIVITIES OF HEPARINOIDS AND HEPARIN

Sample	K_{app} (M^{-1})	$c_{1/2}$ $(\mu g/mL)^a$
S-chitin I	1.4×10^7	0.85
S-chitin II	2.5×10^8	0.20
S-chitin III	7.7×10^8	0.075
SCM-chitin I	5.0×10^5	200
SCM-chitin II	1.0×10^9	0.025
SCM-chitin III	1.9×10^9	0.010
P-chitin		600
CM-chitin		no effect
HE-chitin		no effect
Heparin	1.4×10^9	0.010

[a]Heparinoid concentrations at half inhibition.

conditions. Charged and uncharged chitin derivatives, such as CM-chitin, phosphorylated chitin (P-chitin) and *O*-(hydroxyethyl)chitin (HE-chitin) inhibit thrombin activity little under the general conditions used, although P-chitin showed very slight inhibition at much higher concentration. A similar extent of inhibition was shown by SCM-chitin I.

The apparent binding-constants, calculated by double-reciprocal plots, are listed in Table II, together with minimum concentrations required to decrease activity to half that for thrombin. A similar, apparent binding-constant of SCM-chitin III with thrombin was shown for heparin.

S-Chitin III, which is the most highly sulfated sample, showed 13% of the activity of SCM-chitin III on the inhibition of thrombin action. Although a cooperative effect of the carboxyl and sulfate groups in SCM-chitin is suggested to be a driving force for the inhibition of thrombin activity, SCM-chitin I shows a

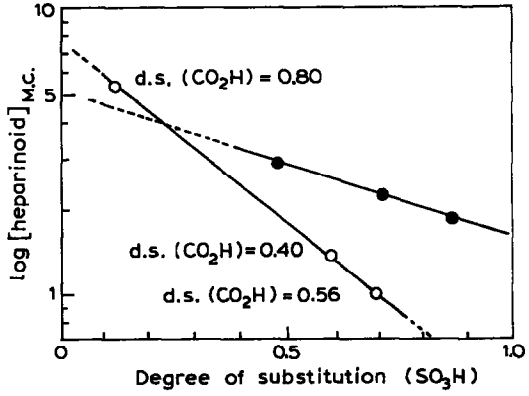

Fig. 3. Relationship between the concentration for 50% inhibition of anticoagulant activity of heparinoids and the degree of sulfation.

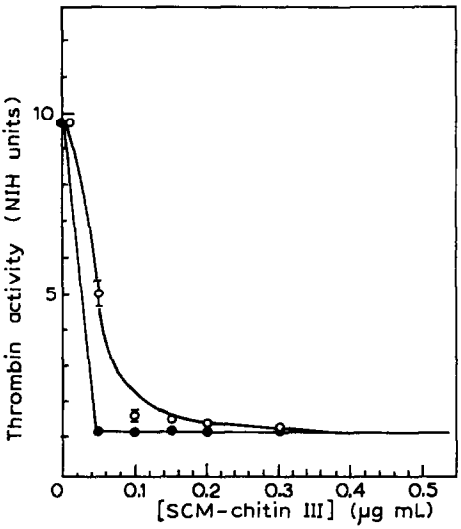

Fig. 4. Inhibitory action of AT-III–heparin (—●—) and AT-III–SCM-chitin III (—○—) on the transformation of fibrinogen into fibrin by thrombin at pH 7.4 and room temperature.

much lower degree of inhibition than the S-chitins. The sulfate group on the GlcNAc residue appears to be the main factor in regulating the anticoagulant activity, because this activity is enhanced by an increase in the sulfate content (Fig. 3). Anticoagulant activity is augmented by the introduction of carboxymethyl groups into S-chitin when a satisfactory level of sulfate is present.

SCM-Chitin III inhibited thrombin activity almost as much as heparin. As the major process of antithrombogenic activity is achieved through the AT-III–heparin complex, the contribution of AT-III was examined with the use of SCM-chitin III, as shown in Fig. 4.

Preliminary results showed only a 50% increase in inhibition of fibrin clotting as compared with heparin; evidently there is significant participation of *N*-sulfate groups, in addition to O-sulfate and carboxyl groups, for heparinoid activity.

EXPERIMENTAL

Materials. — Chitin was prepared from Queen Crab shells by the method of Hackman[11] and powdered to 45–60 mesh before use. Reagents, of reagent grade, were obtained from Wako Pure Chemical Industries, and used without furher purification. Bovine fibrinogen was purchased from Seikagaku Kogyo Co., as a 95% clottable preparation (lot no. 100). Heparin, sodium salt, of 159 I.U./mg (lot M9R8164) was purchased from Nakarai Chemical Industries. Bovine thrombin was supplied from Mochida Pharmaceutical Co. having 1,000 NIH units per vial (lot 3B428). Bovine plasma AT-III was purchased from Boehringer Mannheim (lot 1443301).

Preparation of heparinoids. — Variously carboxymethylated chitins were prepared from chitin according to previous methodology[12]. The degrees of substitution used were 0.40, 0.56, and 0.80. The general procedure for sulfation was that of Horton and Just[6]: 2 g of chitin or CM-chitin was treated 3 times with 70 mL of distilled pyridine to remove water and suspended in 70 mL of pyridine. Chlorosulfonic acid–pyridine (10 mL of $ClSO_3H$ in 60 mL of distilled pyridine) was added to the chitin suspension and the mixture was boiled under reflux for 90 min with stirring. The supernatant liquid was decanted off and the residue, suspended in ice–water, was adjusted to pH 9 with 2M NaOH. The precipitate formed by the addition of ethanol was redissolved in water, dialyzed against deionized water to remove free salt, and subsequently lyophilized; yield: 1.3–3.4 g (50–80%). The degree of sulfation was estimated by quantitative analyses for sulfur in the products[13].

Apparent molecular weight of heparinoids. — Molecular weights were estimated from viscosity measurements using an Ubbelohde-type viscometer by applying the equation proposed for heparin[14]. The molecular weights of CM-chitins were also estimated by the viscosity equation proposed by Inoue *et al.*[15].

Clottability. — Bovine fibrinogen was dissolved in deionized water and adjusted to 0.5 mg/mL as determined from the absorbance density at 280 nm (1% = 1.506) according to Mihalyi[16]. Thrombin was dissolved in 10 mL of 5mM Tris–HCl buffer (pH 7.4) to give an activity of 100 units/mL. Thrombin was first pretreated with heparin or heparinoids of various concentrations for 5 min at 25° and pH 7.4 (5mM Tris–HCl buffer containing 5mM $CaCl_2$ and 0.1M KCl). The final activity of thrombin was 10 NIH units/3.7 mL. Fibrinogen solution (1.5 mL) was added the mixture to start the clotting and the mixture was agitated gently with a glass hook until the first appearance of fibrin threads, as described in ref. 17. The clotting time was plotted against the dilution of standard thrombin to determine the remaining thrombin activity in the mixture. The thrombin activity was standarized by the rate of hydrolysis of the TAME HCl. For measuring the effect of AT-III on the anticoagulant activity of heparin or SCM-chitin III, preincubation of AT-III (10 NIH units) with heparin or SCM-chitin III for 5 min was performed before mixing with thrombin.

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