Time-Dependent Conformational Change of Thrombin Molecules Induced by Sulfated Polysaccharides

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Dextran sulfate (DS) had a greater ability to elute thrombin adsorbed on a small Sepharose 6B column than heparin did, while chondroitin sulfate A had little ability. It is probable that the strength of the interaction of thrombin depends mostly on the charge-density of strongly acidic sulfate groups in the polysaccharides. The change in intrinsic fluorescence intensity of thrombin with time was closely correlated with the rate of inactivation of the enzyme in the presence of sulfated polysaccharides. Both rates were affected by the pH of the solution in the presence of the polyanions. The rates in the presence of DS were highest at pH 6.05 among the three pHs tested, while they were enhanced only at pH 6.05 by heparin, but not by chondroitin sulfate A. Therefore, extensive charge-neutralization of thrombin by the sulfated polysaccharides is able to induce time- and temperature-dependent intramolecular conformational change (irreversible denaturation) of the enzyme molecules.

Keywords thrombin; antithrombin; heparin; dextran sulfate; chondroitin sulfate; hyaluronate

Introduction

Thrombin interacts with heparin[1)] and with dextran sulfate (DS)[1g,j,2)] in various ways. Since association of heparin with thrombin is mostly electrostatic,[1n,3)] disparate values of their association constants have been reported, probably due to determination under different conditions.[1p,s,v)] Moreover, some measurements were done in systems consisting of three components (enzyme, sulfated polysaccharides and inhibitor or substrate).[1u,4)] Since antithrombin III (AT III, inhibitor) and fibrinogen (substrate) also interact with sulfated polysaccharides,[1g,j,s,5)] it was difficult to identify which component predominantly interacted with the sulfated polysaccharides. Previous results suggested that thrombin has a qualitatively higher affinity for DS than for heparin.[1j)]

Thrombin is tightly adsorbed on the surface of laboratory were as well as several gel matrices.[2b-d,6)] DS prevented thrombin from being adsorbed on Sepharose 6B, and was able to release the enzyme adsorbed on the gel in the cold.[2b-d)] Moreover, DS enhanced inactivation of thrombin in dilute solution at 37 °C.[2b)] These phenomena can provide useful information on the affinities of thrombin for the sulfated polysaccharides.

Intrinsic fluorescence of proteins is due to the presence of aromatic amino acids, mostly tryptophan.[7)] The observed protein fluorescence is based on a delicate balance of interacting forces, and it is extremely sensitive to changes in the local environment of the tryptophan residues. The marked sensitivity of the various parameters of fluorescence (quantum yield, life time of the excited state, polarization, *etc.*) to chemical and physical changes in the environment makes fluorescence measurement one of the mostly useful methods for studying the structure and conformational changes of protein molecules. However, there is a serious problem in that quenching, defined as the reduction in the quantum efficiency of a fluorophore due to interaction with other solutes,[8)] may be due to either complex formation between a protein and solute or interception of energy transfer, as with some inorganic salts. Thus, fluorescence quenching of a protein molecule can only be assumed to be due to conformational change if such a change is confirmed by other physical methods or if there is a stoichiometric interaction.

It is known that the fluorescence intensity of trypsin decreases with denaturation.[9)] Previously, I reported time-dependent decrease in the intensity of trypsin and fibrinogen fluorescence in dilute solution under thermo-controlled conditions.[5a,10)] The decrease of trypsin fluorescence was closely correlated with the rate of inactivation of the enzyme.[10)] Moreover, no measurable degradation of proteases in dilute solution was observed during the inactivations.[11)] These facts suggest that the time-dependent change in the fluorescence intensity of these proteins is due to denaturation (irreversible conformational change) of the molecules. Thus, measurement of time-dependent change in the protein fluorescence may be a convenient method to determine the interaction with other solutes.

This paper describes irreversible conformational change of thrombin molecules in the presence of sulfated polysaccharides. The sulfated polysaccharide-enhanced conformational change of the enzyme molecules depended on the pH of the solution and on the charge-density of the strongly acidic sulfate groups on polysaccharides.

Materials and Methods

Materials Pig intestinal heparin (168 USP units/mg, sulfur content = 12.2%) and DS (8 kilodalton (kDa), sulfur content = 17%) were from Sigma Chemical Co., St. Louis, MO. Sodium hyaluronate (rooster comb) and chondroitin sulfate A (sulfur content = 6.4%) were from Seikagaku Kogyo Co., Ltd., Tokyo, and Nakarai Chemical Co., Kyoto, respectively. *tert*-Butoxycarbonyl-Val-Pro-Arg-4-methylcoumaryl-7-amide (Boc-Val-Pro-Arg-MCA) was from the Protein Research Foundation, Minoh, Osaka. Sepharose 6B and another DS (500 kDa, sulfur content = 17%) were obtained from Pharmacia Fine Chemicals AB, Uppsala. Toluidine Blue O was from E. Merck AG, Darmstadt.

Reported methods were used for preparation, activation and purification of bovine thrombin.[1j,12)] The purified protein appeared homogeneous on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Concentrations of thrombin on weight and molar bases were determined spectrophotometrically using $A_{1cm}^{1\%} = 19.5$ and a molecular weight of 37000.[1j)]

Purified heparin (11 kDa, 184 USP units/mg and sulfur content = 12.2%) free from dermatan sulfate, and heparin preparations with high and low affinities for AT III (HA- and LA-heparins, 227 and 9 USP units/mg, respectively) prepared by the reported method[13)] were kind gifts from Prof. K. Nagasawa of this University. No significant difference in sulfur contents of HA- and LA-heparins was observed from that of the original material. Those preparations were used only in some experiments in Table I.

Chromatography on a Small Column Sepharose 6B (settled volume, 3 ml) in a disposal syringe (1.3 × 2.3 cm) with a Nylon cloth at the bottom were equilibrated with 0.1 M NaCl and 0.1 M Tris-HCl (pH 8.3). Thrombin

(25 pmol) was applied to the column and washed with 10 ml of the buffer. The adsorbed thrombin was eluted with 1 ml of sulfated polysaccharide (44 μg) and then further eluted with the buffer. Fractions of 2.5 ml/tube were collected at a flow rate of 20 ml/h at 4 °C. In another study, the washed column was developed with 50 ml of a linear gradient of 0 to 20 μg/ml of heparin or 0 to 2 μg/ml of DS in the same buffer. In the last experiment, the column was developed successively with 2 ml of several polysaccharides (2.6 mg) and then further eluted with the same buffer. All of the tubes used contained NaCl solution to give a final concentration of 0.4 M.

Measurement of Fluorescence Tris–acetate solutions used were prepared as follows: acetic acid was added dropwise to a Tris solution to obtain the desired pH, and the concentration of Tris was adjusted with water. The measurement was carried out by method described previously.[5a,10] The reaction mixture contained 100 nM thrombin with or without sulfated polysaccharide in 3 ml of 50 mM Tris–acetate in a quartz cell. Fluorescence intensity was monitored by pulse irradiation for 5 s at appropriate intervals at 37 °C to avoid possible photooxidation. The first-order rate constant, k', of change in the fluorescence intensity was determined by least-squares analysis of the initial reaction rates, assuming that the maximal decrease of intensity was to 64% of that of the native protein.

Measurement of Activity The amidolytic activity of thrombin was measured fluorometrically as described previously.[1j] The reaction mixture contained thrombin and 40 μM Boc–Val–Pro–Arg–MCA in 1 ml of 0.1 M Tris–HCl (pH 8.0) and 0.2 M NaCl, unless otherwise mentioned.

To measure inactivation of thrombin, the enzyme (final concentration, 1 nM) was incubated with or without sulfated polysaccharide in 1 ml of 50 mM Tris–acetate at the indicated pH in a polypropylene tube for various periods at 37 °C. Residual thrombin activity was measured as described above, unless otherwise mentioned.

Content of sulfated polysaccharides in the eluate was determined by utilizing metachromasis, as described previously.[14]

Results and Discussion

As reported previously,[2b,c] more than 80% of thrombin applied was nonspecifically adsorbed on Sepharose 6B. The adsorbed thrombin was eluted successively from the column with heparin and then with DS in one experiment, and with DS and then with heparin in another experiment. Most of the adsorbed thrombin was eluted with DS (Fig. 1), and the remaining thrombin was eluted with heparin.

On the other hand, only a small amount of adsorbed thrombin was eluted when heparin was applied first, while most of the remaining thrombin was eluted with DS applied next. In agreement with reports that inactivation of proteases in dilute solution and its enhancement by DS were temperature-dependent and observed only in the absence of NaCl,[2b,c,10,11b] no measurable time-dependent inactivation of the enzyme was detected during the chromatography at 4 °C. Since NaCl at high concentration prevented adsorption of thrombin on the surface of laboratory ware,[2b,c,11b] nonspecific adsorption of the enzyme should have been minimal in the collecting tubes containing a final concentration of 0.4 M NaCl. Moreover, since the sulfated polysaccharide did not interfere with measurements of the thrombin activity in the presence of 0.2 M NaCl at pH 8.0,[1j,11b] recovery of protein from the columns could be arising from spontaneous inactivation of the enzyme.

Since much more thrombin was recovered in the first elution with DS than with heparin, it is likely that thrombin interacted more strongly with the former than the latter polysaccharide. This was supported by the result of linear gradient elution with the polyanions (Fig. 2): almost the same amount of thrombin was eluted with DS as with ten times the concentration of heparin. The asymmetric peaks

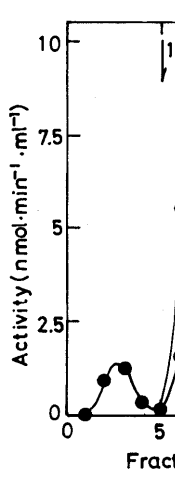

Fig. 1. Elution with Heparin and DS of Thrombin Adsorbed on Sepharose 6B

Bovine thrombin (25 pmol) was applied to a Sepharose 6B column (3 ml settled volume) and washed with 0.1 M NaCl in 0.1 M Tris–HCl (pH 8.3). Adsorbed thrombin was successively eluted at arrows 1 and 2 with 1 ml of DS (44 μg) and then the buffer and with 1 ml of heparin (44 μg) and then the buffer (●), or with heparin (44 μg) and then the buffer, and with DS (44 μg) and then the buffer (○), respectively.

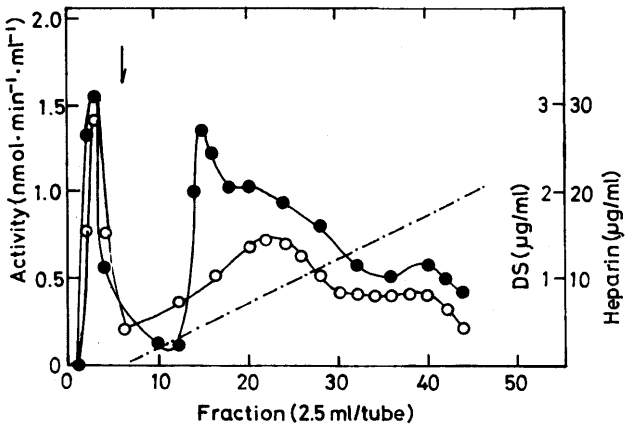

Fig. 2. Linear Gradient Elution by Polysulfates of Thrombin Adsorbed on Sepharose 6B

Thrombin (50 pmol) was applied to a Sepharose 6B column and washed with 0.1 M NaCl in 0.1 M Tris–HCl (pH 8.3). The column was developed with 50 ml of a linear gradient of 0 to 20 μg/ml of heparin (●) or 0 to 2 μg/ml of DS (○) in the same buffer from the arrow. Content of polysaccharides in eluate was determined by utilizing metachromasia of Toluidine Blue O.

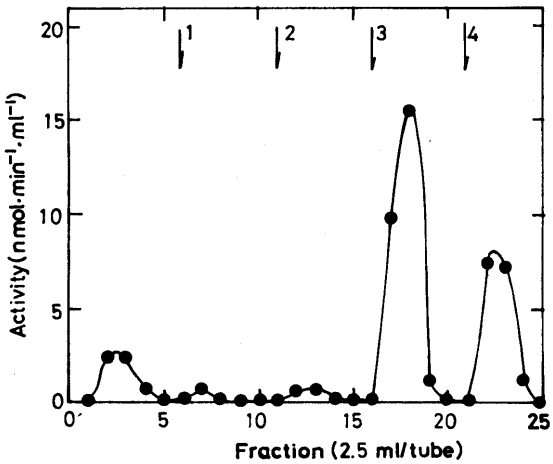

Fig. 3. Successive Elution with Mucopolysaccharides of Thrombin Adsorbed on Sepharose 6B

Thrombin (50 pmol) was eluted successively with 2 ml of each 2.6 mg of sodium hyaluronate, chondroitin sulfate A, heparin and DS in 0.1 M NaCl in 0.1 M Tris–HCl (pH 8.3) at arrows 1, 2, 3 and 4, respectively.

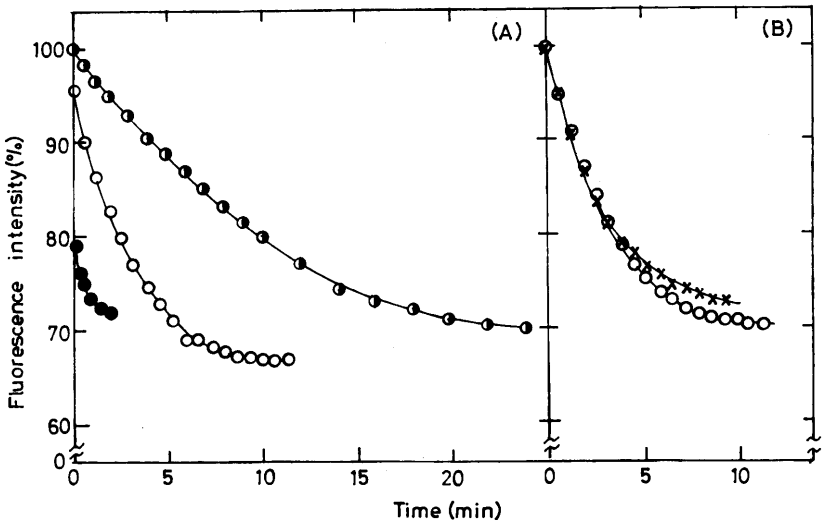

Fig. 4. Change in Intensity of Intrinsic Fluorescence of Thrombin in the Presence of DS

A) The fluorescence intensity of thrombin (100 nM) with 200 nM DS (500 kDa) in 3 ml of 50 mM Tris–acetate (pH 8.0, ◑, pH 7.4, ○, and pH 6.05, ●) was measured at the indicated intervals at 37 °C. The fluorescence intensity of enzyme without DS was taken as 100%. B) The fluorescence intensity with 12.5 μM DS (8 kDa, ×) or 200 nM DS (500 kDa, ○) in 50 mM Tris–acetate (pH 7.4) was measured at the indicated intervals at 37 °C. The fluorescence intensity of enzyme at zero time was taken as 100%.

of the thrombin activity eluted by the polyanions suggested the presence of various thrombin species with different affinities for the polyanions. However, heterogeneous peaks of thrombin activity were observed during gradient elution with salts.[2c] Therefore, broad and asymmetric peaks of the thrombin activity are probably due to heterogeneous gel matrices with different affinities for the enzyme.

To compare the affinities of thrombin for naturally occurring mucopolysaccharides, the column was developed successively with hyaluronate, chondroitin sulfate A, heparin and DS (in increasing order of charge-densities of strongly acidic residues) (Fig. 3). While hyaluronate and chondroitin sulfate A (each 2.6 mg) eluted only 1.8% and 3.5% of total thrombin activity recovered from the column, heparin eluted 53% of the activity. Since 18.6% and 53% of the thrombin activity were recovered with heparin at 44 μg/ml and 2.6 mg/2 ml, respectively, the amount of thrombin eluted increased with increasing concentration of the polyanion, but was not proportional to it. The results in Fig. 3 indicate that thrombin has a higher affinity for heparin than for chondroitin sulfate A, and for DS than for heparin. Since the charge-densities due to carboxylate were roughly the same in the naturally occurring mucopolysaccharides, they appeared to little contribution to the affinities for thrombin (little enzyme was eluted from the column with hyaluronate). Therefore, the differences in affinity for thrombin depended on differences in the charge-densities of strongly acidic sulfate residues on the sulfated polysaccharides.

Previously, I reported that the intensity of intrinsic fluorescence of trypsin and fibrinogen decreased time- and temperature-dependently.[5a,10] Moreover, no proteolysis was observed in DS-enhanced inactivation of thrombin and in solid surface-catalyzed inactivation of chymotrypsin in dilute solution.[11] Thus, apparent time- and temperature-dependent inactivation of thrombin should be due to irreversible conformational change of thrombin molecules. To confirm this, fluorescence measurement was done in a thrombin–polyanion interacting system. As shown in Fig. 4A, the intensity of intrinsic fluorescence of thrombin decreased with time at 37 °C in the presence of DS. Change in the fluorescence intensity of thrombin was almost instantaneous at pH 6.05, but slowest at pH 8.0 in the

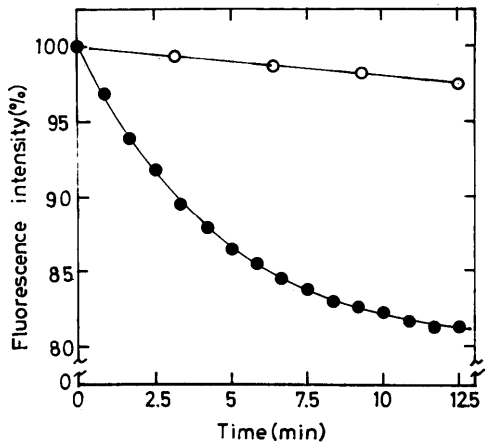

Fig. 5. Change in Fluorescence Intensity of Thrombin in the Presence of Heparin

The fluorescence intensity of 100 nM thrombin with 9.1 μM heparin in 3 ml of 50 mM Tris–acetate (pH 7.4, ○ and pH 6.05, ●) was measured at the indicated intervals at 37 °C.

TABLE I. First-Order Rate Constant of Change in Fluorescence Intensity of Thrombin in the Presence of Heparin

Heparin (μM)	k' (h^{-1})[a]
0	0.86
0.09	8.32±1.18 (n=6)
0.91	7.10±0.33 (n=4)
9.1	7.83±0.12 (n=4)
91	8.77±0.11 (n=3)
9.1[b]	9.74±0.30 (n=3)
9.1[c]	13.32±0.29 (n=3)

a) The constant was determined by least-squares analysis of the initial changes, assuming that the intensity decreased maximally by 36%. *b*) HA-heparin. *c*) LA-heparin.

presence of DS. Double-reciprocal plots of the change *vs.* time gave the maximum decrease to 64% of the original intensity of the native enzyme molecules at pH 8.0 (data not shown). Rates of change in the fluorescence intensity of thrombin were comparable in the presence of 8 kDa DS and 500 kDa DS indicating little effect of molecular size of the polyanions on the affinity for the enzyme (Fig. 4B). At pH 7.4, the fluorescence intensity was constant in the presence of heparin, while it decreased at pH 6.05 (Fig. 5). Since thrombin should be more cationic at pH 6.05 than at pH 7.4, the result confirmed the mostly electrostatic interaction of the enzyme with the sulfated polysaccharides.[1v,3] The rate of change in fluorescence intensity of thrombin did not depend on the concentration of heparin used (Table I), although the standard error increased with decreasing concentration of the polyanion. This was probably due to almost complete saturation of thrombin by heparin, and thus indicated an almost stoichiometric interaction at pH 6.05 in the absence of NaCl. The rate was slightly higher in the presence of HA-heparin, but significantly higher in the presence of LA-heparin. Stronger association of thrombin with LA-heparin than with HA-heparin was also observed in equilibrium gel permeation chromatography.[15] Chondroitin sulfate A was unable to increase the rate of change in fluorescence intensity of thrombin under the conditions used (data not shown).

To confirm that the time-dependent change in the fluorescence intensity of thrombin was mostly due to irreversible conformational change of the enzyme, the effect of pH on the enhancement by sulfated polysaccharides of inactivation of the enzyme was investigated. As shown in Fig. 6, the rate of the thrombin inactivation increased markedly with decrease of pH value in the presence of DS. Since the thrombin activity inhibited by DS was partially restored time-dependently at high concentrations of NaCl,[11b] it was measured in the presence of 25 mM NaCl in this experiment. At pH 6.05, the kinetics of the thrombin inactivation were second order. The result obtained was consistent with the almost instantaneous decrease in the fluorescence intensity of thrombin at that pH. On the other hand, heparin was unable to enhance the inactivation at pH 8.0, confirming the previous report (Fig. 7).[2b] Moreover, while heparin was unable to enhance the thrombin inactivation at pH 7.4, it significantly enhanced that at pH 6.05. The results obtained confirmed those on the change in the fluorescence intensity of thrombin in the presence of sulfated polysaccharides. Heparin somewhat protected thrombin from inactivation at pHs 8.0 and 7.4. Since solid surface seemed to catalyze inactivation of thrombin in dilute solution,[2d] and heparin slightly reduced the affinity of the enzyme for Sepharose 6B (Figs. 1—3), the protective effect of heparin may be due to the reduced affinity of the enzyme for the surface of the polypropylene tubes. All of the results obtained indicate that interaction of thrombin with sulfated polysaccharides increases with acidification, because of increasing net positive charge of the protein molecules, and that thrombin interacts more strongly with DS having a high charge-density of strongly acidic sulfate groups than with heparin at the same pH. Chondroitin sulfate A was unable to enhance the thrombin inactivation even at pH 6.05 (data not shown). Since DS almost instantaneously perturbed trypsin and probably also thrombin,[2c,10] the rates of inactivation and change in fluorescence intensity of thrombin reflect the stability and irreversible conformational change of the enzyme molecules perturbed by the sulfated polysaccharides, but do not reflect the perturbation itself arising from the direct interaction. Therefore, those two methods are adequate to compare the affinity of a protein for several kinds of sulfated polysaccharides, but inadequate to compare that of a sulfated polysaccharide with various kinds of proteins. However, the slight perturbation by heparin on thrombin molecules at pHs 8.0 and 7.4 clearly indicated stronger interaction of the enzyme with DS than with heparin at those pHs.

Assuming that formation of a ternary complex (thrombin–polyanion–AT III or heparin cofactor II) is essential to enhance the protease-inhibitor reaction,[1d] thrombin did not interact with hyaluronate, keratan sulfate, chondroitin 4- and 6-sulfates and heparan sulfate II.[1r,16] More-

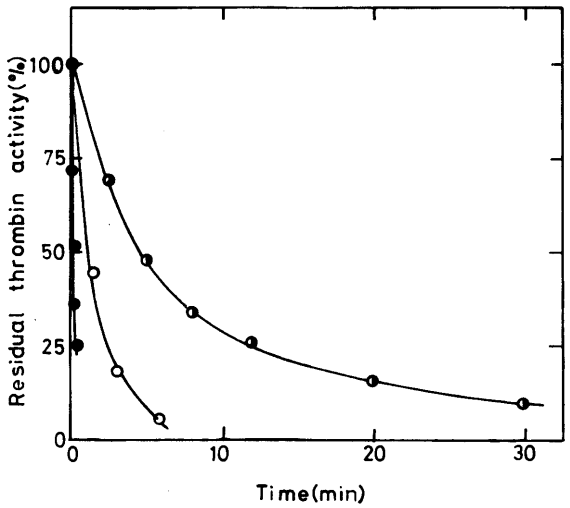

Fig. 6. Effect of pH on Inactivation of Thrombin in the Presence of DS

The reaction mixture containing 1 nM thrombin without or with 12.5 μM DS (8 kDa) in 1 ml of 50 mM Tris–acetate (pH 6.05, ●, pH 7.4, ○ and pH8.0, ◑) was incubated in a cell for the indicated periods at 37 °C. Residual thrombin activity in the cell was measured fluorometrically by adding 25 μl of a mixture of the substrate and NaCl (final concentrations, 40 μM and 25 mM, respectively). At zero time, thrombin and the mixture of the substrate and NaCl were simultaneously added to a buffer with or without DS in a cell.

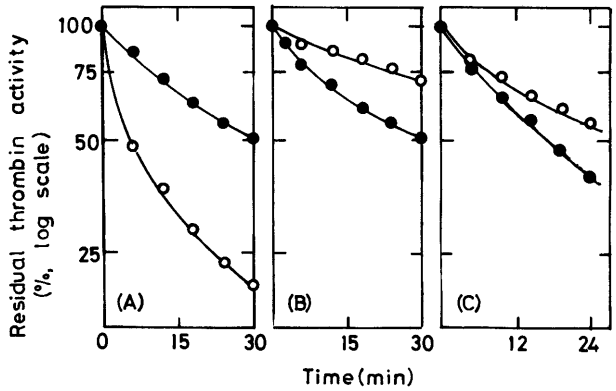

Fig. 7. Effect of pH on Inactivation of Thrombin in the Presence of Heparin

The reaction mixture containing 1 nM thrombin without (●) or with 9.1 μM heparin (○) in 1 ml of 50 mM Tris–acetate (pH 6.05, A, pH 7.4, B, and pH 8.0, C) was incubated for the indicated periods at 37 °C.

over, chondroitin sulfate, dermatan sulfate and one sample of heparan sulfate had little affinity for immobilized thrombin, while heparin and the other heparan sulfate did interact with the enzyme.[11] In the present work at pH 6.05, thrombin interacted strongly and weakly with DS and heparin, respectively, whereas it did not interact with chondroitin sulfate A. The results are consistent with those reported by others. Therefore, measurements of rates of either inactivation of thrombin and/or change in its fluorescence intensity may serve as useful methods to determine directly the interaction of a protein with many polyanions. The stronger perturbing effect of DS than heparin on several protein molecules even at physiological pH may reflect the more toxic actions of the former *in vivo*.[17]

Acknowledgement I thank Professor Kinzo Nagasawa of Kitasato University for encouragement during this work.

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