Comparison of the Inhibition of Thrombin by Three Plasma Protease Inhibitors†

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ABSTRACT: Human α-thrombin is inhibited by the circulating protease inhibitors α_1-antitrypsin, antithrombin III, and α_2-macroglobulin. Kinetic analyses of the inhibitor thrombin interactions were carried out utilizing either fibrinogen or the synthetic substrate Bz-Phe-Val-Arg-*p*-nitroanilide as substrates to determine residual thrombin activity. These studies demonstrated that the inhibition of thrombin by α_1-antitrypsin, antithrombin III, and α_2-macroglobulin followed second-order kinetics. The rate constants for the inhibition of thrombin by α_1-antitrypsin, antithrombin III, and α_2-macroglobulin are $6.51 \pm 0.38 \times 10^3$, $3.36 \pm 0.34 \times 10^5$, and $2.93 \pm 0.02 \times 10^4$ M^{-1} min^{-1}, respectively. Comparison of the second-order rate constants and the normal plasma levels of the three inhibitors demonstrates that, under the in vitro conditions utilized, antithrombin III is five times and α_2-macroglobulin is one-third as effective as α_1-antitrypsin in the inhibition of thrombin.

Of the circulatory proteins normally found in plasma, a number of protease inhibitors have been proposed as playing vital roles in inflammation, coagulation, and fibrinolysis. At least six serine protease inhibitors are found in high concentrations in normal plasma (Heimburger, 1975); of these six, α_1-antitrypsin, antithrombin III, and α_2-macroglobulin are found in high molar concentrations.

Earlier studies have demonstrated that, in patients with a congenital deficiency of antithrombin III, there is a high incidence of thromboembolic disease (Egeberg, 1965a,b, Høie, 1970; Abildgaard, 1971; Marciniak et al., 1974). Antithrombin III has been the subject of extensive investigation demonstrating that the interaction of thrombin with the inhibitor is a 1:1 stoichiometric reaction resulting in the formation of a covalent complex requiring the active site serine of thrombin (Rosenberg & Damus, 1973) and is dissociated by hydroxylamine (Owen, 1975). The inhibitory spectrum of α_2-macroglobulin is very broad in that it appears to interact with and inhibit a variety of proteinases (Barrett & Starkey, 1973) including thrombin (Lanchantin et al., 1966; Shapiro & Anderson, 1977), plasmin (Sugihara et al., 1971), and kallikrein (Harpel, 1971). α_2-Macroglobulin has been suggested as the major plasmin inhibitor in the circulatory system (Nilehn & Ganrot, 1967; Harpel, 1975).

Of the protease inhibitors in plasma, α_1-antitrypsin is found in the highest molar concentration. Attention has been focused primarily on the inhibition of trypsin and chymotrypsin with very few reports as to the effect of α_1-antitrypsin on thrombin. A series of conflicting reports has stated that α_1-antitrypsin either would not (Pannell et al., 1974; Heimburger, 1975; Learned et al., 1976) or would inhibit thrombin (Rimon et al., 1966; Gans & Tan, 1967; Downing et al., 1976, 1977; Matheson & Travis, 1976).

Recent reports by Downing et al. (1976, 1977) have demonstrated that the inhibition of human α-thrombin by α_1-antitrypsin is a 1:1 stoichiometric reaction resulting in the formation of a thrombin-α_1-antitrypsin complex. Under conditions of excess thrombin both the thrombin-α_1-antitrypsin complex and free α_1-antitrypsin are subject to proteolysis. The proteolysis of the free α_1-antitrypsin results in inactivation of the inhibitor. Matheson & Travis (1976) have also studied the interaction of thrombin and α_1-antitrypsin. In these studies it was reported that 1 mol of inhibitor was capable of inhibiting up to 10 mol of thrombin, an observation not consistent with a 1:1 molar interaction.

We have carefully evaluated the kinetics of the inhibition of thrombin by α_1-antitrypsin, antithrombin III, and α_2-macroglobulin. A direct comparison of the kinetics of thrombin inhibition by the three inhibitors was obtained and the relative effectiveness of thrombin inhibition by α_1-antitrypsin, α_2-macroglobulin, and antithrombin III was determined.

Experimental Section

Materials. Human factor IX concentrate, as a lyophilized powder, was obtained from the American Red Cross,[1] Bethesda, Md.; NIH standard thrombin, lot B-3, was obtained from the National Institutes of Health. The synthetic substrate S2160[2] (Bz-Phe-Val-Arg-*p*-nitroanilide) was obtained from Ortho Diagnostics, Raritan, N.J. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was purchased from Sigma Chemical Co., St. Louis, Mo. Aminohexyl-Sepharose was purchased from Pharmacia Fine Chemicals, Piscataway, N.J.

Bovine factor X was prepared according to the procedure of Bajaj & Mann (1973) and activated to factor Xa as previously described (Downing et al., 1975). Bovine factor V was prepared as described by Esnouf & Jobin (1967). Beef lung heparin (1000 units per mL) was obtained from Upjohn, Kalamazoo, Mich.

Proteins. Human thrombin was prepared from factor IX concentrates by a modification of the method described pre-

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[1]Factor IX concentrate, prepared by the American National Red Cross National Fractionation Center and funded by Grant HL-13881-07, was kindly supplied by Dr. Yu-lee Hao of the American National Red Cross, Bethesda, Md.

[2]Abbreviations used: S2160, the synthetic substrate Bz-Phe-Val-Arg-*p*-nitroanilide; Bz, benzoyl; $NaDodSO_4$, sodium dodecyl sulfate.

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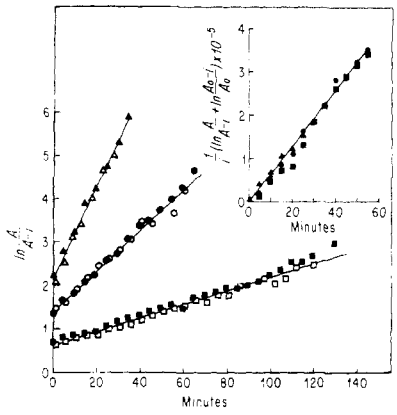

FIGURE 1: Time course of inactivation of thrombin by α_1-antitrypsin. The $\ln(A(A-i)^{-1})$ portion of the second-order rate expression, representing the slope of the line obtained not taking into account the intercept function of the equation, is plotted vs. time. The inset of this figure represents the complete second-order rate expression plotted vs. time and illustrates that the inactivation of thrombin by α_1-antitrypsin follows second-order kinetics with an intercept of zero. The solid symbols represent data collected utilizing the fibrinogen clotting assay to determine the residual thrombin activity and the open symbols represent the inactivation of thrombin as determined by S2160 hydrolysis. The experiments presented are of various ratios of α_1-antitrypsin to thrombin; (■ and □) 2.0 to 1.0; (● and ○) 4.0 to 1.0; and (▲ and △) 8.0 to 1.0.

viously (Downing et al., 1975). The activation of prothrombin in factor IX concentrates to thrombin was carried out in 0.0168 M imidazole, 0.15 M NaCl, 6 mM $CaCl_2$ (pH 7.4) at 37 °C. Twenty units of factor V and 10 units of factor Xa were added per vial of concentrate in the presence of phospholipid. When the level of thrombin activity reached a maximum, the mixture was dialyzed against 0.025 M sodium phosphate (pH 6.5) for purification of the thrombin on sulfopropyl-Sephadex (Lundblad, 1970). α_1-Antitrypsin was prepared by a modification of the method of Pannell et al. (1974). The modifications consisted of fractionation of outdated human plasma between 55% and 72% with ammonium sulfate followed by albumin removal by Cibacron-Blue Sepharose chromatography (Travis et al., 1976) and DEAE-cellulose chromatography at pH 8.8 and 6.5. The trypsin inhibitory activity of α_1-antitrypsin was determined as described by Dietz et al. (1974) utilizing active site titrated trypsin (Chase & Shaw, 1967). The activity of α_1-antitrypsin preparations varied between 85% and 90%. Human antithrombin III was a gift of Dr. Whyte Owen (University of Iowa) and human α_2-macroglobulin was graciously provided by Dr. Margaret Hunter (Institute of Science and Technology, University of Michigan). Protein concentrations were determined spectrophotometrically at 280 nm and corrected for Rayleigh light scattering. Extinction coefficients ($E_{280}^{1\%}$) utilized in concentration determinations were: 19.5 for thrombin (Mann, 1976), 4.84 for α_1-antitrypsin (Bloom & Hunter, 1978), 6.1 for antithrombin III (Miller-Anderson et al., 1974), and 8.7 for α_2-macroglobulin (Harpel, 1976).

Platelet factor 4 was isolated from human platelets and coupled to aminohexyl-Sepharose utilizing 1-ethyl-3-(3-dimethylaminopropyl)carbiodiimide (Khoory et al., 1978).

Assays. Thrombin clotting assays were carried out according to the standard NIH procedure as modified by Mann et al. (1971). S2160 assays were carried out as described by Downing, et al., (1977).

Electrophoresis. A modification of the Swank & Munkres (1971) $NaDodSO_4$ electrophoretic technique, described previously (Butkowski, 1973; Downing et al., 1975) was utilized to determine protein purity and assess the interactions of α_1-antitrypsin with thrombin. Alkaline disc gel electrophoresis (Davis, 1964) was also utilized to assess the homogeneity of protein preparations.

Determination of Thrombin Inhibition. The inhibitory effect of α_1-antitrypsin, antithrombin III, and α_2-macroglobulin on thrombin was determined by assay of residual thrombin activity by both the fibrinogen clotting and S2160 hydrolysis assays. Appropriate concentrations of thrombin and the inhibitor being studied were incubated in 0.02 M Tris, 0.15 M NaCl (pH 7.4) at 37 °C. In all cases the thrombin concentration was held constant at either 0.98 μM or 2.44 μM and the inhibitor concentration varied as desired.

Determination of Second-Order Rate Constants. The equation describing a second-order process is given by:

$$v = kAB \tag{1}$$

where v is the velocity of the reaction, k is the second-order rate constant, and A and B are the concentrations of inhibitor and enzyme, respectively. Integration of eq 1 results in the expression given by Jencks (1969):

$$\frac{1}{B_0 - A_0} \ln \left[\frac{A_0(B_0 - x)}{B_0(A_0 - x)}\right] = kt \tag{2}$$

where A_0 and B_0 are the initial inhibitor and enzyme concentrations, x is the concentration of enzyme–inhibitor complex, and t is time. Substituting $A_0 - A$ for x and i for $A_0 - B_0$, eq 2 can be rewritten to give:

$$\frac{1}{i}\left[\ln\left(\frac{A}{A-i}\right) + \ln\left(\frac{A_0 - i}{A_0}\right)\right] = kt \tag{3}$$

The residual enzyme (thrombin) activity is directly determined and A_0 and B_0 are known; thus the amount of free inhibitor (A) can be calculated from:

$$A = A_0 - (B_0 - B) \tag{4}$$

Thus, a plot of $\ln[A/(A-i)]$ vs. time yields a linear plot with a slope of ki.

Results

Purification of Proteins. The procedures utilized in the preparation of human α-thrombin and α_1-antitrypsin yielded highly purified protein preparations. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and alkaline disc gel electrophoresis demonstrated that the α-thrombin, α_1-antitrypsin, and antithrombin III preparations were homogeneous.

Kinetics of the Inhibition of Thrombin by α_1-Antitrypsin. The inhibition of thrombin by α_1-antitrypsin results in the formation of a high molecular weight complex. The molecular weight of the complex suggested that there was a 1:1 molar stoichiometry involved in the inhibition of thrombin. If one molecule of α_1-antitrypsin interacts with and inhibits one molecule of thrombin, then the process should be a second-order process. Extensive kinetic analysis of the inactivation of thrombin by α_1-antitrypsin, utilizing both S2160 hydrolysis and fibrinogen clotting assays to determine residual thrombin activity, demonstrates that the inhibition of thrombin follows second-order kinetics. When the concentration of α_1-antitrypsin is twice that of thrombin, a plot of the slope function of eq 3 vs. time yields a straight line with a slope equal to ki as is illustrated in Figure 1. As the concentration of α_1-antitrypsin is doubled, the rate of inhibition is doubled and, when the inhibitor concentration is eight times that of thrombin, the rate of inhibition is four times that observed at a twofold excess of inhibitor (Figure 1). Linear regression analysis of the data

TABLE I: Second-Order Rate Constants Determined for the Inhibition of Thrombin by α_1-Antitrypsin.[a]

Thrombin to α_1-antitrypsin ratio	Assay procedure (M^{-1} min^{-1})	
	S2160 hydrolysis	Fibrinogen clotting
1:8	6.75×10^3	6.02×10^3
1:4	6.28×10^3	6.80×10^3
1:2	6.58×10^3	6.94×10^3
1:1	6.00×10^3	6.27×10^3
2:1		7.03×10^3

[a]The calculation of second-order rate constants for the inhibition of thrombin by α_1-antitrypsin under conditions in which thrombin was in excess of the inhibitor was complicated by the fact that there were two competing reactions: first, the inhibition of thrombin; and second, the proteolysis and inactivation of α_1-antitrypsin by free thrombin in the system. As the concentration of thrombin was increased relative to that of the inhibitor, there was extensive degradation of the inhibitor. Because of the rapid inactivation of α_1-antitrypsin it was not possible to determine meaningful second-order rate constants after the concentration of thrombin was increased above twice that of α_1-antitrypsin.

TABLE II: Second-Order Rate Constants Determined for the Inhibition of Thrombin by α_2-Macroglobulin and Antithrombin III.

Inhibitor	Thrombin to inhibitor ratio	Assay procedure (M^{-1} min^{-1})	
		S2160 hydrolysis	Fibrinogen clotting
α_2-Macroglobulin[a]	1:4		2.92×10^4
	1:2		2.95×10^4
Antithrombin III	1:4		3.0×10^5
	1:2		3.69×10^5
	1:2		3.43×10^5
	1:1	3.21×10^5	3.83×10^5
	1:1	3.05×10^5	

[a]Utilizing S2160 hydrolysis to determine residual activity, there was never greater than a 50% loss of thrombin activity at α_2-macroglobulin concentrations up to eight times higher than that of thrombin. The determination of the second-order rate constant was taken from the data obtained over the initial 15 min of the reaction of concentrations of α_2-macroglobulin two and four times that of thrombin.

presented in Figure 1 yields intercepts that are within 10% of the theoretical values. Replotting the data presented in Figure 1 in the format of the complete second-order rate expression (eq 3) yields a linear plot with an intercept of zero (inset of Figure 1). The second-order rate constant for the inactivation of thrombin by α_1-antitrypsin was calculated to be $6.51 \pm 0.38 \times 10^3$ M^{-1} min^{-1} (Table I).

Kinetics of α_2-Macroglobulin and Antithrombin III in the Inhibition of Thrombin. Because the inhibition of thrombin by α_1-antitrypsin is a second-order reaction, it was desirable to determine the kinetics of the inhibition of thrombin by α_2-macroglobulin and antithrombin III such that a direct comparison of thrombin inhibitions could be made. Several concentrations of each inhibitor with thrombin were studied. Thrombin inhibition by α_2-macroglobulin, as determined by S2160 hydrolysis and fibrinogen clotting assay, led to an observation similar to that described for trypsin inhibition (Rinderknecht et al., 1975). Assay of residual thrombin activity by S2160 hydrolysis, at various molar concentrations of α_2-macroglobulin in excess of thrombin, resulted in the observation that never more than 50% of the original activity was inhibited. However, in utilizing the fibrinogen clotting assay, it was observed that up to 90% of the original thrombin activity could be inhibited when the α_2-macroglobulin was present at four times the concentration of thrombin. Varying the ratio of α_2-macroglobulin to thrombin from a fourfold excess of inhibitor to an equimolar concentration of inhibitor to thrombin decreased not only the rate but also the extent of thrombin inhibition. It is apparent that the thrombin-α_2-macroglobulin complex is still active toward low molecular weight substrates but not to the macromolecular substrate fibrinogen.

When fibrinogen was used as the thrombin substrate to determine residual activity in the presence of α_2-macroglobulin, an apparent second-order rate constant of $2.93 \pm 0.02 \times 10^4$ M^{-1} min^{-1} (Table II) was calculated for the initial phase of thrombin inhibition. Reliable kinetic data could not be obtained at α_2-macroglobulin to thrombin ratios of less than 2 to 1.

Inhibition of thrombin by antithrombin III was a more straightforward process. Several inhibitor-thrombin concentrations were studied by following the residual thrombin activity both by S2160 hydrolysis and fibrinogen clotting assays. In all cases, the inhibition of thrombin was both rapid and complete with the rate of inhibition increasing as the concentration of antithrombin III was increased relative to that of thrombin. The second-order rate constant for the inhibition of thrombin by antithrombin III was calculated to be $3.36 \pm 0.34 \times 10^5$ M^{-1} min^{-1} (Table II).

Because the isolation of antithrombin III involved affinity chromatography on heparin-Sepharose, there was the possibility that there were trace amounts of heparin in the antithrombin III preparations. Heparin is an accelerator of the inhibition of thrombin by antithrombin III and for these studies it was imperative that there be no heparin in the reaction mixtures which would confuse the kinetic analysis of thrombin inhibition by antithrombin III. In order to assess the potential heparin contamination in the antithrombin III preparations, platelet factor 4 was coupled to Sepharose; antithrombin III was then subjected to chromatography on this platelet factor 4 Sepharose. The antithrombin III eluting from the platelet factor 4 Sepharose column was then utilized to determine a rate constant for the inhibition of thrombin. The second-order rate constant after chromatography was 3.05×10^5 M^{-1} min^{-1}, essentially identical with the rate constant obtained before chromatography. To assess the capability of platelet factor 4 Sepharose in removing heparin, a second experiment was carried out. Antithrombin III (0.79 mg, 12.2 nmol) was treated with heparin (50 units); the second-order rate constant for the inhibition of thrombin could not be determined because there was virtually instantaneous inhibition of all thrombin activity under our experimental conditions. The antithrombin III-heparin mixture was then subjected to platelet factor 4 Sepharose chromatography. A second kinetic determination utilizing the antithrombin III eluted from the column resulted in a second-order rate constant of 2.9×10^5 M^{-1} min^{-1} for the inhibition of thrombin.

Comparison of the Efficiency of α_1-Antitrypsin, Antithrombin III, and α_2-Macroglobulin in the Inhibition of Thrombin. A direct comparison of the effectiveness of α_1-antitrypsin, antithrombin III, and α_2-macroglobulin in the inhibition of thrombin was made by evaluation of the second-order rate constants for the inactivation of thrombin by each inhibitor. The direct comparison of the inhibition of thrombin by all three inhibitors is presented in Figure 2, where the complete second-order rate expression (eq 3) is plotted. It

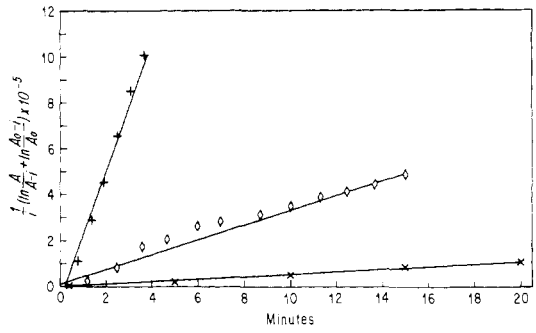

FIGURE 2: Comparison of the inactivation of thrombin by α_1-antitrypsin (X), α_2-macroglobulin (◊), and antithrombin III (+) as represented by the complete second-order rate expression. Residual thrombin activity was determined by the fibrinogen clotting assay in all experiments in which the concentration of the inhibitor was four times that of thrombin.

is apparent that thrombin inhibition by antithrombin III and α_1-antitrypsin follows second-order kinetics. The inhibition of thrombin by α_2-macroglobulin exhibits mixed function kinetics. These kinetics, however, will be treated as second order for purposes of comparison. From the data presented, it is apparent that antithrombin III is a much more effective inhibitor of thrombin than is α_2-macroglobulin or α_1-antitrypsin and α_2-macroglobulin is more effective than is α_1-antitrypsin. Direct comparison of the rate constants demonstrated that, on a mole per mole basis, α_2-macroglobulin is 4.5 times more effective and antithrombin III is 51.6 times more effective than α_1-antitrypsin in the inhibition of thrombin.

The normal plasma levels of the three inhibitors in plasma are reported to be 42.4 μM for α_1-antitrypsin, 4.46 μM for antithrombin III, and 3.11 μM for α_2-macroglobulin (Kueppers & Black, 1974). If one considers the plasma level of each inhibitor in relation to the second-order rate constant for the inhibition of thrombin, it becomes apparent from the second-order rate constants presented in Table III that antithrombin III is 5.16 times more effective than α_1-antitrypsin and α_2-macroglobulin is only one third as effective as α_1-antitrypsin in the inhibition of thrombin in vitro.

Discussion

The inhibition of thrombin by α_1-antitrypsin occurs via the interaction of the two proteins in a 1:1 stoichiometric ratio. This was suggested by earlier observations (Downing et al., 1976, 1977) in which the apparent molecular weight of the thrombin-α_1-antitrypsin complex approximated the sums of the apparent molecular weight of thrombin and α_1-antitrypsin. In a report by Matheson & Travis (1976) it was noted that their preparations of α_1-antitrypsin and α-thrombin interacted in such a manner that as much as 10 mol of thrombin was inhibited by 1 mol of α_1-antitrypsin. This investigation, however, has demonstrated that the inactivation of thrombin by α_1-antitrypsin follows second-order kinetics, an observation which is consistent with a 1:1 molar stoichiometry between inhibitor and protease. Thrombin inhibition in the presence of excess α_1-antitrypsin resulted in linear plots for the second-order rate expression with intercepts near theoretical values. Similar analysis of initial rate data from experiments in which thrombin was four times more concentrated than the inhibitor resulted in second-order rate constants of the same order of magnitude as those presented in Table I; however, at later times the kinetics became complex. As the concentration of thrombin was increased, the evaluation of the data became more difficult, since there was extensive proteolysis and inactivation of α_1-antitrypsin by thrombin.

TABLE III: Comparison of the Relative Thrombin Inhibitory Effectiveness of α_1-Antitrypsin, Antithrombin III, and α_2-Macroglobulin.

Inhibitor	Plasma concn (μM)[a]	2nd-order rate constant (M^{-1} min^{-1})	Rel effectiveness as a thrombin inhibitor
α_2-Macroglobulin	3.11	2.93×10^4	1
α_1-Antitrypsin	42.4	6.51×10^3	3.03
Antithrombin III	4.46	3.36×10^5	15.64

[a]Calculated from data given by Kueppers & Black (1974).

Like the inactivation of thrombin by α_1-antitrypsin the inactivation by antithrombin III follows second-order kinetics. The kinetics of thrombin inhibition by antithrombin III become complex under conditions in which large molar excesses of thrombin are incubated with the inhibitor, presumably due to proteolysis of the inhibitor. Because heparin potentiates the inhibition of thrombin by antithrombin III, it was important to exclude the possibility of heparin contamination of antithrombin III preparations which had been isolated utilizing heparin affinity chromatography. Insolubilized platelet factor 4 was shown to be effective in removing all of the heparin from antithrombin III which had been treated with heparin. In addition, antithrombin III which had not been treated with heparin was subjected to platelet factor 4 Sepharose chromatography. The results from these experiments demonstrated that the original antithrombin III preparations were free of heparin contamination in that there was no change in the second-order rate constant for the inhibition of thrombin after platelet factor 4 Sepharose chromatography.

Reported normal plasma concentrations of α_2-macroglobulin are similar to those reported for antithrombin III. Shapiro & Anderson (1977) reported that in plasma which is allowed to clot a large fraction of radiolabeled thrombin is associated with α_2-macroglobulin whereas in purified systems composed of α_2-macroglobulin, antithrombin III and radiolabeled thrombin the majority of the thrombin was bound to antithrombin III. It was of interest to directly compare the ability of α_2-macroglobulin with other protease inhibitors in order to assess its potential role in the inhibition of thrombin. Although the kinetics of α_2-macroglobulin inactivation of thrombin were complex and not strictly second order, it is possible to utilize the apparent second-order rate constant obtained in this study for comparative purposes.

Under the in vitro conditions utilized in this investigation and considering only the second-order rate constants, α_2-macroglobulin and antithrombin III were 4.5 and 51.6 times more effective than α_1-antitrypsin in the inhibition of thrombin. However, if the reported normal plasma molar concentrations of all three inhibitors are also considered the relative thrombin inhibitor effectiveness of antithrombin III was calculated to be 15 times more effective than α_2-macroglobulin and α_2-macroglobulin only one-third as effective as α_1-antitrypsin.

Acknowledgments

The authors wish to thank Dr. Whyte Owen and Dr. Margaret Hunter for providing the antithrombin III and α_2-macroglobulin utilized in this study.

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