INHIBITION OF THE ENZYMATIC ACTIVITY OF THROMBIN BY CONCANAVALIN A

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SUMMARY: Concanavalin A, a carbohydrate lectin derived from the jack bean, prolongs the thrombin clotting time of human plasma or purified fibrinogen. Prolongation is due to delay in peptide release from fibrinogen. The rate of fibrin monomer polymerization is not affected. Hydrolysis of protamine sulfate by thrombin is inhibited by concanavalin A. All inhibitory effects are prevented by α-methyl-D-mannoside. Concanavalin A does not delay clotting of fibrinogen by reptilase (releases fibrinopeptide A only) or by Ancistrodon contortrix contortrix (releases fibrinopeptide B initially followed by a small amount of A). It is concluded that concanavalin A binds to a carbohydrate on the thrombin molecule thus inhibiting its enzymatic activity.

Preliminary studies in this laboratory have shown that concanavalin A* prolongs the partial thromboplastin time, prothrombin time and thrombin times of human plasma (1). Prolongation is prevented by α-methyl-D-mannoside+ which is known to bind to Con A (2). The effect of Con A on the thrombin time has been studied in detail to elucidate the mechanism of prolongation. Three possible mechanisms were considered: 1) Con A binds to thrombin obscuring the active site, 2) Con A binds to fibrinogen inhibiting peptide release, and 3) Con A binds to fibrin monomer inhibiting polymerization. Results obtained indicate that Con A inhibits the enzymatic activity of thrombin by binding to a saccharide on the molecule.

MATERIALS: The following were obtained from the Sigma Chemical Company: Concanavalin A (Grade IV highly purified), purified bovine thrombin (substantially free of other clotting factors and plasmin), protamine sulfate Grade III from herring (clupeine), hirudin Grade II. General Biochemicals was the source of α-methyl-D-mannoside, α-methyl-D-glucoside, β-methyl-D-glucoside, β-methyl-D-xylopyranoside, and 3-O-methyl-D-glucoside. Reptilase was obtained from Pentapharm, Basel; Ancistrodon contortrix contortrix from the Miami Serpentarium, fluorescamine (fluoran) from Roche Diagnostics and glycine methylester hydrochloride from Mann Research Laboratories.

*Con A +α-D-Man

TABLE I

PROLONGATION OF THE THROMBIN FIBRINOGEN CLOTTING TIME BY CON A AND CORRECTION BY α-D-MAN

Fibrinogen 2 mg/ml 2.5 mM Na Citrate and 144 mM NaCl	Thrombin 4 units/ml 0.2 M Phosphate buffer pH 7.5	Con A 2 mg/ml 144 mM NaCl	α-D-Man 2.8 mg/ml 144 mM NaCl	144 mM NaCl	Clotting Time secs
0.1 ml	0.1 ml	-	-	0.2 ml	16
0.1 ml	0.1 ml	0.1 ml	-	0.1 ml	40
0.1 ml	0.1 ml	0.1 ml	0.1 ml	-	16
0.1 ml	0.1 ml	-	0.1 ml	0.1 ml	15

Clotting times were measured at 37°C. Values are representative of 10 experiments. α-D-Man was preincubated with Con A at 37°C for 2 minutes prior to use.

METHODS AND RESULTS: Clotting of Fibrinogen by Thrombin As shown in Table I, Con A prolongs the clotting time of purified human fibrinogen (96% clottable) by purified bovine thrombin, and α-D-Man completely prevents this prolongation. The following other sugars were tested in similar concentration and did not prevent prolongation by Con A: β-methyl-D-glucoside, β-methyl-D-xylopyranoside, 3-O-methyl-D-glucoside, α-methyl-D-glucoside. Con A also prolonged the thrombin clotting time of normal human plasma and α-D-Man prevented prolongation.

Kinetic studies were carried out on the thrombin fibrinogen clotting time using differing concentrations of fibrinogen and Con A. Results shown in Figure 1 show a Km of 1.9×10^{-6}M for fibrinogen clotting (mol. wt. 356,000 [3]). Con A appeared to act as a competitive inhibitor with a Ki of 1.5×10^{-5}M (mol. wt. 55,000 [2]).

Effect of Con A upon the Release of Fibrinopeptides from Fibrinogen by Thrombin The release of fibrinopeptides was measured by a fluorescent assay using fluorescamine (4). Fibrinogen, 2 mg in 0.2 ml of 2.5 mM sodium citrate and 144 mM NaCl was incubated at 37°C with 2.5 units thrombin in 0.1 ml of 0.2 M phosphate buffer pH 7.4. The reaction was stopped and fibrinogen precipitated with 0.3 ml absolute ethanol. Samples were left in an ice bath for one hour, and then centrifuged to obtain a supernatant of which an 0.02 aliquot was added to 1.2 ml of 0.2 M phosphate buffer pH 7.4. While mixing vigorously, 0.4 ml of fluorescamine (0.1 mg/ml in dried acetone) was added. Fluorescence was determined on an American Instrument Company SPF - 125 fluorometer at an excitation wavelength of 390 nm, and emission wavelength of 470 nm. Parallel experiments were run with Con A, 2 mg/ml and Con A plus α-D-Man, 8 mg/ml. Results are expressed in Figure 2. These indicate that Con A inhibits release of fibrinopeptides from purified fibrinogen and that α-D-Man prevents this inhibition.

Fibrinopeptide release was also studied by a method described in detail by Forman et al. (5). Thrombin and fibrinogen were incubated together at pH 5.2. Under these conditions fibrinopeptide release occurred but the fibrin monomer formed did not polymerize. The pH was then raised to 7.2 to permit polymeriza-

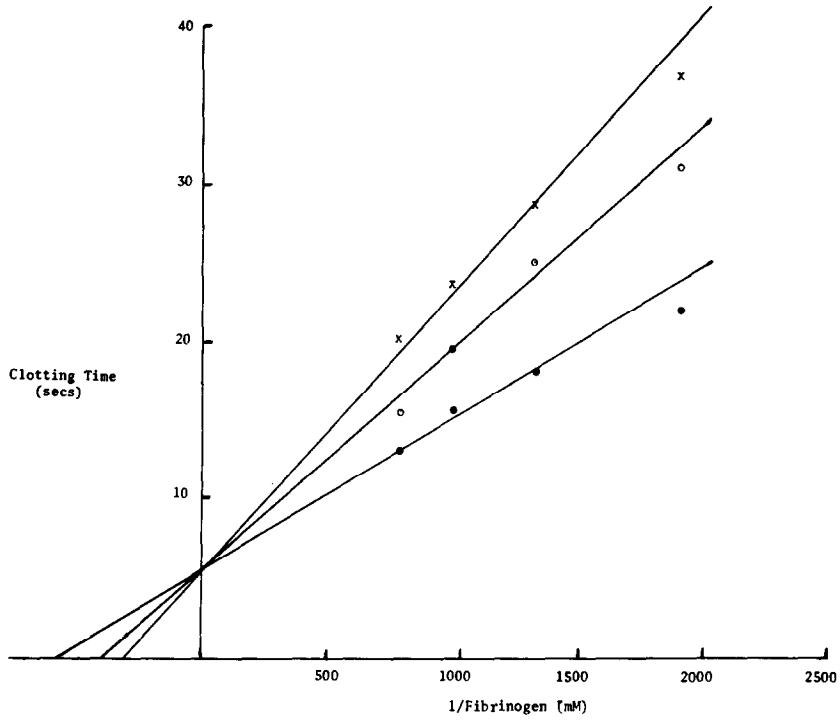

Figure 1. Kinetic study of the effect of Con A on the fibrinogen-thrombin clotting time. Fibrinogen concentration was varied in a final volume of 0.3 ml and mixed with various reagents at a final concentration of 2 u/ml thrombin, 0.8 mM Na Citrate, 97 mM NaCl, 70 mM Phosphate buffer, pH 7.5. Symbols refer to: •, no additions; o, Con A, 0.17 mg/ml; x, Con A, 0.33 mg/ml. When Con A 0.33 mg/ml was preincubated with α-D-Man 0.66 mg/ml at 37°C for 2 minutes, values identical with the control were obtained.

tion and formation of a visible clot, in the presence of hirudin 2 mg/ml which prevents further fibrinopeptide release. Inclusion of 1 mg/ml Con A in the low pH mixture inhibited release of fibrinopeptides, as measured indirectly by rate of clot formation. This inhibition was prevented by the presence of α-D-Man at 1.4 mg/ml (10 experiments).

The Effect of Con A on Fibrin Polymerization Fibrin monomer was prepared from purified human fibrinogen. The method described by Donnelly et al. (6) was used with the addition of glycine methylester hydrochloride (7). 0.1 ml of soluble fibrin monomer in 1 M NaBr at pH 5.3 was allowed to clot by adding it to 1 ml of 0.05 M phosphate buffer pH 6.0. Inclusion of Con A from 1 to 5 mg/ml final concentration did not affect the speed of clot formation (5 experiments).

Polymerization was also studied as per Forman et al. (5) utilizing hirudin.

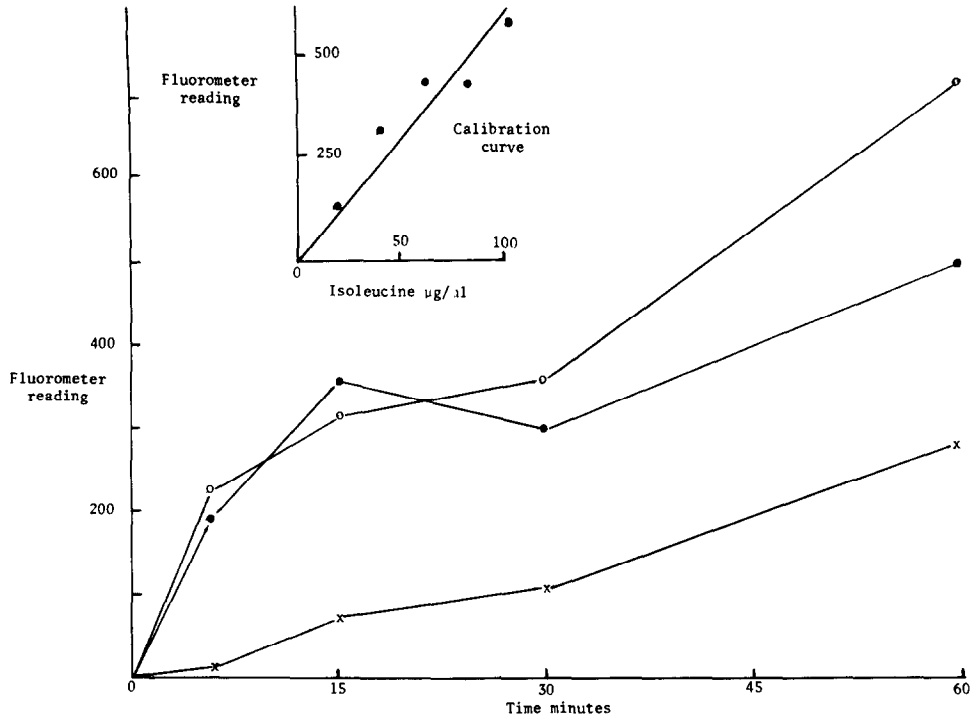

Figure 2. Fluorescamine assay of effect of Con A on fibrinopeptide release at pH 7.4. See Methods for detail. Calibration curve is given as insert. Data represent the average of 3 experiments. Symbols refer to: •, control; x, Con A, 2 mg/ml; o, Con A, 2 mg/ml plus α-D-Man, 8 mg/ml.

On this occasion Con A (1 to 10 mg/ml) was added with the hirudin (2 mg/ml) when the pH was raised to allow polymerization. There was no delay in polymerization (4 experiments).

Effect of Con A upon the Clotting Time of Fibrinogen by Reptilase and by Ancistrodon Contortrix Contortrix Clotting times were performed using 2.5 mg/ml purified fibrinogen and 4.0 mg/ml Reptilase which releases fibrinopeptide A only; or 2 mg/ml fibrinogen and 2 mg/ml Ancistrodon contortrix contortrix in 10^{-3}M $CaCl_2$. This venom releases fibrinopeptide B initially followed by a small amount of A (8). The presence of 2 - 4 mg/ml Con A did not prolong the clotting times (6 experiments).

Effect of Con A on the Hydrolysis of Protamine Sulfate by Thrombin Hydrolysis was quantitated using fluorescamine by the technique described by Brown et al. (9)

TABLE II

EFFECT OF CON A ON HYDROLYSIS OF PROTAMINE SULFATE BY THROMBIN

Protamine 10 mg/ml 0.1 M Phosphate buffer pH 7.5	Thrombin 25 units/ml 0.1 M Phosphate buffer pH 7.5	Con A 0.5 mg/ml 144 mM NaCl	Con A 0.5 mg/ml + α-D-Man 32 mg/ml 144 mM NaCl	144 mM NaCl	Fluorometer reading
0.05 ml	0.05 ml	-	-	0.05 ml	189
0.05 ml	0.05 ml	0.05 ml	-	-	79
0.05 ml	0.05 ml	-	0.05 ml	-	204

Reagents were incubated together at 37°C for 30 minutes before transferring 0.02 ml into 1.2 ml 0.1 M Phosphate buffer pH 7.5. Fluorescamine 0.4 ml was then added with vigorous mixing. Results represent the mean of 18 experiments.

Parallel incubations at 37°C were carried out with 0.1 M phosphate buffer pH 7.5, 10 mg/ml protamine, and 25 u/ml thrombin; protamine, thrombin and Con A; or protamine, thrombin, Con A and α-D-Man. Results which are shown in Table II indicate that Con A inhibits hydrolysis and that α-D-Man prevents this inhibition. Inhibition was best achieved using 0.25-0.5 mg/ml Con A. Inhibition was prevented with relatively higher concentrations of α-D-Man than required to inhibit fibrinopeptide release (16-32 mg/ml).

DISCUSSION: These results indicate that Con A prolongs the thrombin fibrinogen clotting time by inhibiting the enzymatic activity of thrombin and not by altering the fibrinogen molecule. This was suggested by the inhibition of thrombin hydrolysis of protamine with Con A. However, the possibility remained that the fibrinogen molecule was also altered by the lectin. We were able to show that fibrin polymerization was not affected. The delay in fibrinopeptide release could also have been due to binding of Con A to fibrinogen in such a manner as to delay that release. To resolve this possibility, we investigated the effect of Con A on the clotting of fibrinogen by two snake venoms. Reptilase removes only peptide A; polymerization can occur without the release of B. Ancistrodon contortrix contortrix initially releases peptide B, and after a delay a small amount of A appears but clotting does not occur until the release of A. The clotting time with either venom was unaffected by Con A. Thus it appears that the reason for prolongation of the thrombin fibrinogen clotting time by Con A is inhibition of the enzymatic activity of thrombin.

Con A is a lectin with a specific binding site for α-D-Man or α-D-glucopyranoside (2). Inhibition of thrombin activity with Con A and release of this inhibition with α-D-Man but not α-D-glucoside as well as three other sugars suggests that Con A impedes this reaction by binding to α-D-Man on the thrombin molecule.

It is known that thrombin has a carbohydrate residue which is situated on the B chain at position 65-66 and that this contains mannose (10). The active enzymatic site containing aspartic-serine-glycine is also situated on the B chain

but at position 191-202 (10). It is therefore possible that Con A binds at this distant site but still blocks the active site. This would suggest a conformation in which the carbohydrate site is sufficiently close to the active site for Con A to interfere.

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