

Fibrinolytic action on fresh human clots of whole body extracts and two semipurified fractions from *Lonomia achelous* caterpillar

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Abstract

The severe bleeding diathesis produced by intoxication with the venom of *Lonomia achelous* caterpillars is characterized by prolonged bleeding from superficial skin wounds as well as massive hemorrhage into body cavities. The aim of the present study was to evaluate the effect of the crude venom and its fibrinolytic fractions on *in vitro* lysis of whole blood clots. Venom fractions with fibrinolytic activity were obtained by gel filtration chromatography on Sephadex G75 using imidazole buffer, pH 7.4, at a flow rate of 24 ml/h. Four peaks with fibrinolytic activity were obtained by this method. The highest activity was found in the first two peaks (both peaks were used for the experiments). The results show that the caterpillar venom degraded the preformed clots at a slower rate than plasmin. In addition, plasma protease inhibitors of the fibrinolytic system (α_2 -antiplasmin, α_2 -macroglobulin, PAI, etc.) only weakly inhibited the lytic effect of the caterpillar venom. These characteristics, as well as the pattern of fibrinogen degradation products, the delay period on fibrin plate lysis and amidolytic activity on chromogenic substrate, reported previously, indicate that the caterpillar enzymes are different from plasmin and trypsin.

Key words

- *Lonomia achelous* caterpillars
- Fibrinolysis
- Clot lysis
- Plasmin

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This work is part of a
Master's thesis presented by
E. Coll-Sangrona to the
Department of Biochemistry,
Instituto Venezolano de
Investigaciones Científicas.

Received April 8, 1997
Accepted February 6, 1998

Introduction

Contact with caterpillars of the Saturniidae *Lonomia achelous* produces a bleeding syndrome in humans. Laboratory tests carried out on patients show intense fibrinolytic activity (1-4). The hemolymph and hair secretions of the *Lonomia achelous* caterpillar have been partially purified by molecular exclusion chromatography, showing a similar chromatographic elution pattern as measured by UV absorbance and biological activ-

ity (fibrin plate lysis and activity on chromogenic substrates) (5). The venom and the fibrinolytic fractions degrade fibrin(ogen) *in vitro*. However, the digestion products obtained are completely different from the plasmin or trypsin digestion products, indicating a different mode of action (6).

In the present study we have tested the fibrinolytic effect of *Lonomia achelous* caterpillar venom (LACV) on preformed whole human blood clots. Whole body extracts (WBE) of caterpillars and their fibrinolytic

fractions were used at different concentrations. Urokinase (UK), tissue plasminogen activator (t-PA) and plasmin (Pm) were used for comparisons. The results show that the venom and its active fractions degrade human whole blood clots, although at a slower rate than the corresponding enzymes, and are little affected by physiological plasma protease inhibitors.

Material and Methods

Reagents

Bovine thrombin was obtained from Sigma Chemical Co., St. Louis, MO, dissolved in 25 mM CaCl₂ to a final concentration of 6.0 NIH U/ml. Plasmin and single chain tissue plasminogen activator (sc-tPA) were obtained from Kabi Vitrum (Stockholm, Sweden), and used at a final concentration of 10 to 50 U/ml (UK equivalent units). Urokinase, a gift from Mr. R. Hurt of American Diagnostica Inc. (Greenwich, CT), was used at final concentrations ranging from 10 to 100 U/ml. All reagents were prepared immediately before use and kept on ice.

Caterpillar material

The caterpillars were collected in the field and their hemolymph and saliva collected separately. A whole body extract was prepared by maceration of the skin in 0.15 M NaCl/imidazole buffer, pH 7.4, to obtain a protein concentration of 40 mg/ml. Ten ml of the preparation were subjected to molecular exclusion chromatography using Sephadex G75 as described previously (5). The experiments were repeated ten times. Chromatographic fractions were pooled according to protein concentration and fibrinolytic activity and called Fractions I, II, III and IV (FI, FII, FIII, FIV) by order of elution. Protein concentration was measured by the method of Lowry and coworkers (7). Fibrinolytic activity was measured by fibrin plate

lysis (8,9) and by amidolytic activity on chromogenic substrate S-2444 (10). The activity is reported as urokinase equivalents per milliliter (UK U/ml) by comparison with a urokinase standard (American Diagnostica Inc.) curve. For the experiments only WBE and Fractions I and II (which were the ones with good fibrinolytic activity) were used. WBE was used at final concentrations of 10 to 100 UK U/ml and FI and FII at 10 and 25 UK U/ml, due to the low specific activity of these fractions.

Clot formation

Clots were prepared as described by Sabovic (11), with some modifications. Briefly, a plastic syringe was connected to a piece of Tygon™ tubing, and closed with a hemostatic clamp. The syringe was filled with 1 ml of fresh human whole citrated blood. Bovine thrombin at a final concentration of 6.0 NIH U/ml and CaCl₂ at a final concentration of 1.25 mM were added. The blood was mixed three times by inversion, allowed to fill the tubing and left to clot at 37°C for 45 min. At the end of this period the Tygon tubing was cut into 1-cm long pieces using a pre-calibrated device, producing pieces containing approximately 100 µl blood. The clots were expelled into a Petri dish (containing cold 0.15 M NaCl) by means of a syringe and washed several times with cold 0.15 M NaCl until the solution became colorless.

Clot lysis

The washed clots were suspended either in 1 ml 0.15 M phosphate buffer supplemented with 0.1% human albumin (Miles Laboratories Inc., Clifton, NJ), or in 1 ml citrated human plasma containing 2 U/ml of unfractionated heparin (Farma S.A., Caracas, Venezuela). Aliquots of 50 µl of each inductor (UK, Pm, t-PA, WBE, FI or FII) were added to the final concentrations men-

tioned before. To monitor spontaneous lysis, control tubes were prepared using 50 μ l phosphate buffer instead of inductors. Samples were incubated at 37°C in a shaker (Labquake Labindustries, Thomas Scientific, Swedesborn, NJ) at a speed of 18 rpm. At 2, 4, 6 and 24 h, aliquots of the buffer or plasma (until 6 h only) were removed and the hemoglobin concentration was determined by the cyanomethemoglobin technique (12). The amount of hemoglobin liberated in each aliquot (indicative of clot dissolution) was expressed as percentage (% of lysis) of the initial hemoglobin value, that was considered to be 100%. This value was obtained by measuring the hemoglobin concentration present in 100 μ l of a lysate of whole blood in 1.050 μ l of buffer. The experiments were performed 8 times separately. The parametric method for small samples of the Student *t*-test was used for statistical analysis (13).

Results and Discussion

Figure 1 shows the elution pattern of the whole body extract purification by gel filtration chromatography on Sephadex G75. Contrary to what was found with hemolymph purification (5), four peaks with fibrinolytic activity were observed. The highest activity was found in peaks I and II (both peaks were chosen for the experiments). Figure 2 shows the lysis produced by all fibrinolytic enzymes (UK, Pm, sc-tPA, WBE, FI, FII). It should be noted that when the clots were suspended in buffer (Figure 2A) the caterpillar material (WBE, FI and FII) at all concentrations studied caused a biphasic response, characterized by a slow dissolution for the first 6 h followed by an increased lysis rate during the following 24 h. A similar delay was always observed in experiments with chromogenic substrates and fibrin plate lysis using WBE, FI or FII (data not shown). The corresponding enzymes (UK, Pm, t-PA) all showed a uniform linear progressive increase in lysis.

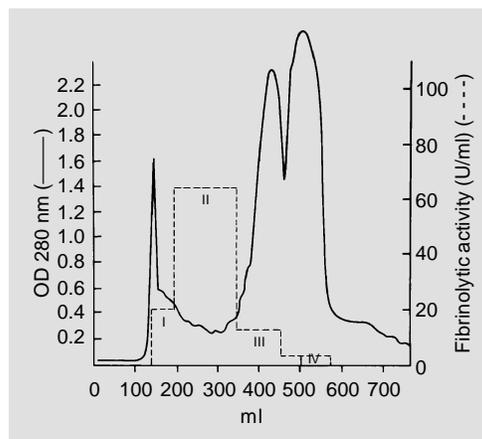


Figure 1 - Chromatographic pattern of whole body extract purification.

WBE and FII at 10 IU/ml concentration exceeded the spontaneous dissolution of the control clots. This difference was not significant ($P > 0.05$) during the first 6 h of incubation. At 24 h, FI showed a degree of clot lysis similar to that produced by t-PA, Pm and UK. As expected, with 25 and 50 IU/ml there was a progressive increase in lysis with all inductors. WBE, FI and FII dissolved the clots at a slower rate, reaching a similar degree of lysis as the other fibrinolytic agents at 24 h incubation. However, WBE at a concentration of 100 IU/ml produced a higher rate of lysis than the control, which became significantly higher ($P < 0.05$) than the control after 2 h of incubation and exceeded the degree of lysis produced by the corresponding enzymes at 24 h.

Arocha-Piñango and collaborators (3,4) have shown that the fibrinogen degradation products (FDP) found in patients intoxicated with LACV do not inhibit the thrombin-fibrinogen reaction since the thrombin time is totally corrected by the addition of normal plasma. Subsequently, they demonstrated (6) that the *in vitro* degradation pattern of fibrinogen produced by the hemolymph and its fibrinolytic fraction was different from the degradation produced by plasmin or trypsin. They postulated that the venom cleaves the fibrinogen molecule at sites different from those cleaved by these enzymes. This could explain the poor inhibitory effect of

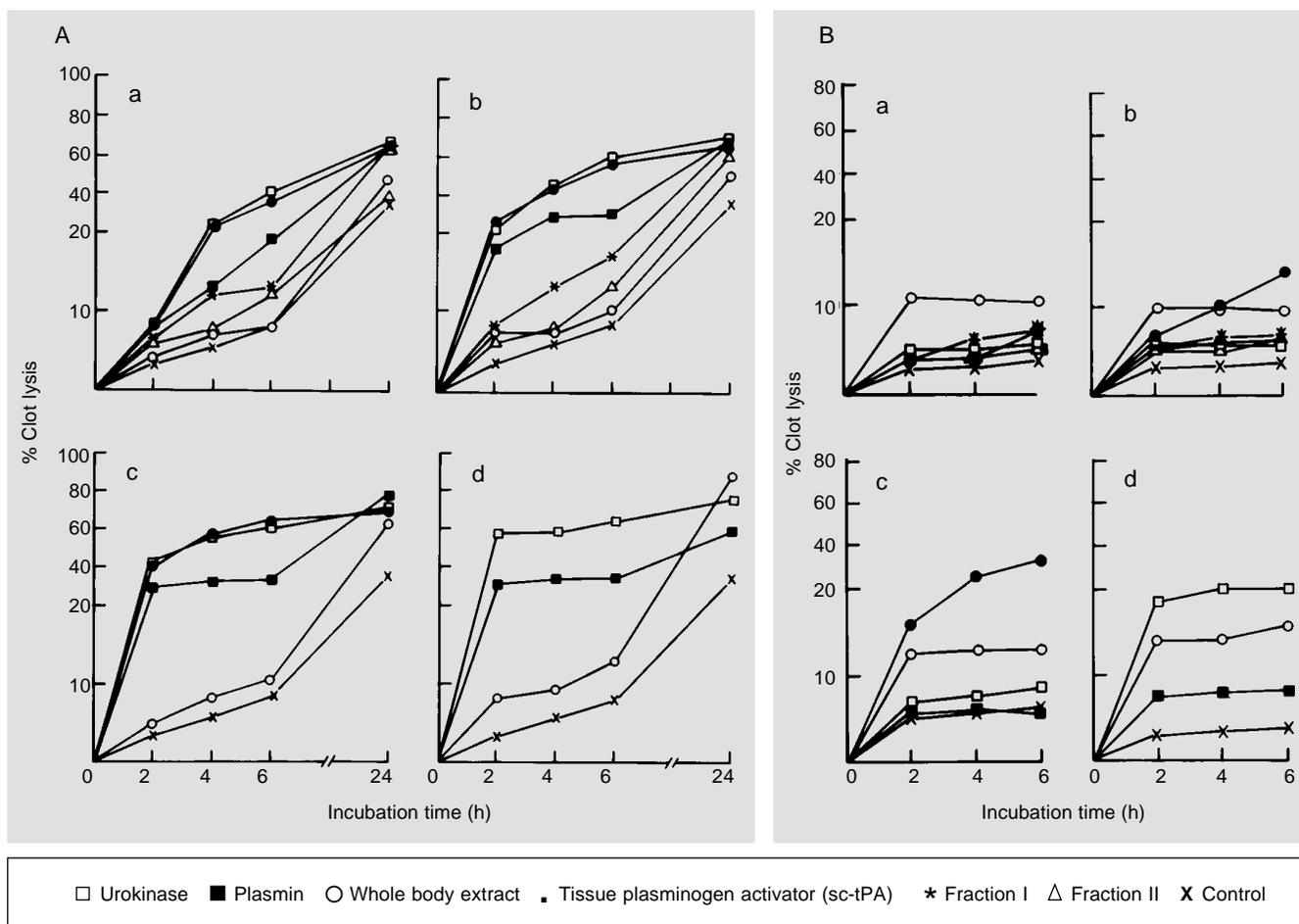


Figure 2 - Pattern of clot lysis. A, Clots suspended in buffer. B, Clots suspended in plasma. Inductor concentrations: a) 10 U/ml, b) 25 U/ml, c) 50 U/ml, d) 100 U/ml.

FDP found in patient plasma (3,4). Previous experiments with *Lonomia achelous* crude hemolymph and chromatographic fractions (10) have demonstrated that they hydrolyze the chromogenic substrates S-2251, S-2444 and S-2288 designed for plasmin, urokinase and t-PA, respectively. These observations suggest the presence of activities similar to Pm, UK and t-PA in the different components of the caterpillar venom. The differences in the velocity of clot dissolution shown in the present paper and in the release of pNA from the chromogenic substrates shown in previous experiments seem to support the hypothesis that the fibrinolytic effect of the caterpillar venom is different from that of the physiological enzymes (14-21).

Several authors have reported a strong inhibition of the fibrinolytic enzymes in plasma due to the presence of circulating plasma inhibitors (17-22). The action of these physiological plasma inhibitors on the fibrinolytic action of the caterpillar venom was evaluated by studying the dissolution of the clot suspended in plasma (Figure 2B and Table 1). A remarkable decrease in lysis was observed when t-PA, Pm and UK were used indicating, as expected, the strong inhibition by the plasma inhibitors. On the other hand, the lytic action of WBE was not inhibited, but rather was enhanced compared with clots suspended in buffer. The lytic action of FI and FII was inhibited by plasma, albeit at a lower proportion than t-PA, Pm and UK.

These data show that the plasma physiological inhibitors do not affect WBE-dependent clot lysis. On the contrary, it seems that there may exist some components in plasma that increase the WBE action, possibly by inducing conformational changes, increasing the substrate interaction or acting as cofactors of the enzymatic activity. It is also possible that WBE contains one or more components that protect the fibrinolytic enzyme from the plasma inhibitors, and that these components are lost during the purification process.

In summary, we demonstrate that the fibrinolytic enzymes present in the WBE of the *Lonomia achelous* caterpillar and its active fractions degrade preformed human blood clots, and that this degradation occurs at a slower rate than that caused by the comparative enzymes. The present results also show that the mammalian plasma inhibitors have a lower effect on the caterpillar venom than on the other activators. This seems to confirm that the action of the caterpillar venom is different from that of the physiological enzymes. These observations, taken together with the fact that FDP from intoxicated patients do not inhibit the thrombin-fibrinogen reaction and that the treatment with antifibrinolytic agents promptly stops the bleeding, even at very low fibrinogen concentrations and high levels of FDP (3,4), have led us to conclude that there exists a potentially useful thrombolytic agent in *Lonomia achelous*. Its delayed action (after 6 h of incubation) would offer the addi-

Table 1 - Percent inhibition of clot lysis by plasma.

| Inductor | 2 h | 4 h | 6 h |
|------------------------------|------|------|------|
| 10 U/ml | | | |
| Tissue plasminogen activator | 48.3 | 81.7 | 79.5 |
| Urokinase | 39.3 | 78.0 | 86.5 |
| Plasmin | 49.4 | 67.9 | 72.3 |
| Whole body extracts | 0 | 0 | 1.13 |
| Fraction I | 34.8 | 46.7 | 44.1 |
| Fraction II | 35.0 | 48.7 | 53.3 |
| 25 U/ml | | | |
| Tissue plasminogen activator | 73.1 | 76.3 | 74.3 |
| Urokinase | 74.0 | 87.2 | 90.1 |
| Plasmin | 69.8 | 79.8 | 79.6 |
| Whole body extracts | 0 | 0 | 11.3 |
| Fraction I | 32.5 | 51.0 | 59.3 |
| Fraction II | 16.9 | 36.0 | 52.2 |
| 50 U/ml | | | |
| Tissue plasminogen activator | 59.1 | 55.3 | 50.0 |
| Urokinase | 83.4 | 86.1 | 85.5 |
| Plasmin | 80.2 | 81.3 | 81.3 |
| Whole body extracts | 0 | 0 | 0 |
| 100 U/ml | | | |
| Tissue plasminogen activator | 67.9 | 66.0 | 68.2 |
| Plasmin | 75.3 | 77.0 | 76.1 |
| Whole body extracts | 0 | 0 | 0 |

tional advantage of sustained thrombolytic support after the induction with some of the short-acting agents commonly used.

Acknowledgments

We thank Dr. Ulf Lundberg for useful comments and assistance with the English version, Z. Carvajal and Mrs. A. Gil for collaboration with the laboratory work and Mrs. L. Rybak for secretarial assistance.

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