THROMBOLECTIN: A LECTIN ISOLATED FROM *BOTHROPS ATROX* VENOM

T. Kent GARTNER, Kurt STOCKER+ and Danny C. WILLIAMS
Department of Biology, Memphis State University, Memphis, TN 38152, USA and *+Pentapharm Laboratories, Basle, Switzerland*

Received 19 May 1980

1. Introduction

Venom from the reptile *Bothrops atrox* can convert plasma fibrinogen into fibrin and induce platelet aggregation [1–3]. Each activity is attributed to a distinct serine protease isolated from the venom: batroxobin [1] (or reptilase) and thrombocytin [2,3]. Purified batroxobin can generate fibrin by hydrolysis of fibrinogen, yet is incapable of causing platelet aggregation [1]. Conversely, thrombocytin induces platelet aggregation, but has very limited, if any, ability to convert fibrinogen into fibrin [2,3]. Here we report that *Bothrops atrox* venom also contains a lectin which we have designated thrombolectin. This lectin is a disulfide-linked, dimeric protein of apparent monomer mol. wt ~15 000. The activity of the lectin is inhibited maximally by β-galactosides and, in contrast to other galactolectins [4], is calcium-dependent and inhibited by reducing agents.

2. Materials and methods

2.1. *Materials*

Thrombocytin, reptilase® and Chromozym TH were supplied by Pentapharm Ltd., Basle. The thrombocytin was purified as in [2]. Fetuin, diisopropyl phosphofluoridate (DFP), thiodigalactoside, and the other sugars were obtained from Sigma Chemical Co. St Louis.

2.2. *Methods*

Thrombocytin amidolytic activity was assayed as in [2] using Chromozym TH as substrate. The erythrocytes were prepared and the microtiter hemagglutination assays run as in [5] except that Tyrode buffer (1 g glucose, 8 g NaCl, 1 g $NaHCO_3$, 0.2 g $CaCl_2$, 0.1 g $MgCl_2 \cdot 6\ H_2O$, 0.2 g KCl, 0.05 g $Na_2HPO_4 \cdot 1\ H_2O$/liter, pH 7.4) was used in the plates. A 10 × molar excess of DFP was added (10–200 μl) to thrombocytin in Tris–imidazole–saline buffer (0.1 M Trizma base, 0.1 M imidazole and 0.15 M NaCl, pH 8.4) [2]. The mixture was incubated at room temperature for 30 min and assayed without dialysis. As a control, thrombocytin was treated the same way except that no DFP was added. Thrombolectin was isolated from *Bothrops atrox* venom (Pentapharm Ltd., Basle) by affinity chromatography using asialofetuin–Sepharose 4B. Fetuin (150 mg) was desialyzed by incubation in 10 ml 0.001 M NaH_2PO_4, 0.15 M NaCl (pH 2.0) at 80°C for 1 h [6]. The resulting asialofetuin solution was dialyzed against saline for 24 h at 4°C. The asialofetuin was coupled to CNBr-activated Sepharose 4B (20 ml packed) prepared by the method in [7]. All isolation procedures were done at 4°C. The asialofetuin–Sepharose 4B column was poured and equilibrated with Tyrode buffer (pH 7.4). Crude venom (200 mg in 4 ml Tyrode buffer) was applied to the column, extraneous materials were removed by washing with Tyrode buffer. Thrombolectin was eluted from the column with 0.05 M lactose in Tyrode buffer. The purified isolated thrombolectin was dialyzed exhaustively against Tyrode buffer at 4°C and stored frozen at −12°C until used. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done as in [8], with a few modifications. SDS–PAGE gel slabs containing thrombolectin were stained for carbohydrate using the periodic acid-Schiff's reagent [9]. Protein determinations were made as in [10].

3. Results

3.1. *Discovery of the hemagglutinin*

In the course of characterizing the expression of the endogenous hemagglutinin [11] of human platelets, we discovered that high (≧20 μg/ml) concentra-

tions of thrombocytin [2,3] agglutinated formaldehyde fixed bovine erythrocytes. This hemagglutination activity was present in each of 3 thrombocytin batches (14/104, 16/104, 17/104) tested with our assay system. In contrast, batroxobin purified from the same venom used to prepare batch 17/104 of thrombocytin, lacked hemagglutination activity. The hemagglutination activity was also present in the crude venom.

3.2. *The hemagglutinin is a lectin*

The hemagglutinin was identified as a lectin by demonstrating that its activity was specifically inhibited by saccharides. Lactose and β-thiodigalactoside (TDG), an analogue of lactose, blocked the activity of the lectin, whereas D-glucose, methyl α-D-mannoside, D-galactosamine, D-glucosamine, D-mannosamine, L-arginine, and L-glutamine had little or no effect on the hemagglutination activity of the thrombocytin preparations.

3.3. *The lectin is not thrombocytin*

The hemagglutination and proteolytic activities of thrombocytin preparations were characterized to determine if thrombocytin was the lectin. Heating the thrombocytin preparation at 65°C for 10 min inactivated >94% of the lectin activity but had no effect on the proteolytic activity (table 1). DFP treatment of the thrombocytin preparation destroyed >97% of the proteolytic activity but had no effect on the lectin activity. Crude venom which had both thrombocytin activity and lectin activity was incubated at room temperature with formaldehyde-fixed bovine erythrocytes. The erythrocytes agglutinated and rapidly settled out. The cell-free supernatant fraction resulting from centrifugation of this material had <3% of the original lectin activity and 100% of the original proteolytic activity. Incubation of the crude venom for the same time under the same conditions except that the buffer lacked fixed erythrocytes resulted in no loss of either activity. Some of the lectin bound to the aggregated fixed erythrocytes was recovered by adding 0.5 M final conc. thiodigalactoside to the resuspended erythrocytes presumably containing the lectin. The cells were removed and the supernatant fraction was dialyzed at 4°C against Tyrode buffer. The dialysate contained hemagglutinin activity with a high titer but lacked protease activity. Trypsin treatment of the thrombocytin preparation destroyed its lectin activity. These results demonstrate that the lectin activity and the proteolytic activity of the thrombocytin preparations were caused by separable proteins.

3.4. *Purification and characterization of the lectin*

The lectin was purified using an asialofetuin-derivatized Sepharose 4B column, a standard technique for purifying galactolectins [6,12] (see section 2). The purified thrombolectin lacked detectable (thrombocytin) amidolytic activity. Fig.1 reveals that thrombolectin had monomer mol. wt ~15 000 on SDS–PAGE in the presence of β-mercaptoethanol. In contrast, under non-reducing conditions the predominant form of thrombolectin was a dimer of mol. wt

Table 1
Characterization of the lectin and proteolytic activities of the thrombocytin preparations

Treatment	% Protease act.	% Lectin act.
Untreated thrombocytin	100	100
Heat-treated thrombocytin	100	≤6
DFP-treated thrombocytin	3	100
Trypsin-treated thrombocytin	–	0

Thrombocytin at 0.25 mg/ml in Tris–imidazole–saline buffer at pH 8.4 was heated at 65°C for 10 min. Trypsin treatment of the thrombocytin was done by adding 10 μl 1.5 mg trypsin/ml solution in Tyrode buffer to 200 μl 0.25 mg thrombocytin/ml solution of in Tris–imidazole–saline buffer. The mixture was incubated at room temperature for 20 min. A 10 × weight excess of soybean trypsin inhibitor was added. As controls, untreated thrombocytin was diluted with Tyrode buffer and another aliquot of thrombocytin received trypsin pretreated with soybean trypsin inhibitor. The trypsin–soybean–trypsin inhibitor mixture did not inactivate the lectin activity of the thrombocytin preparation

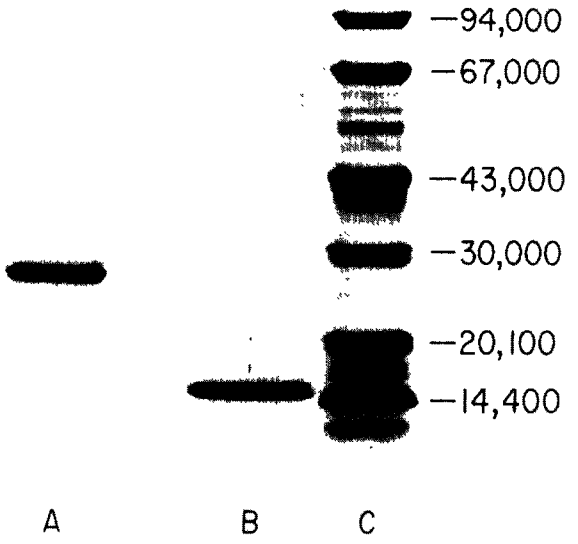

Fig.1. SDS–polyacrylamide gel electrophoresis of thrombolectin. Samples of isolated thrombolectin were resolved on SDS–polyacrylamide gel slabs prepared as exponential gradients of 5–20% acrylamide. (A) contains 7.6 μg non-reduced purified thrombolectin; (B) contains 7.6 μg purified thrombolectin fully reduced with 10% β-mercaptoethanol; (C) contains 6 low molecular weight standards obtained from Bio-Rad Labs: phosphorylase b (M_r 94 000); albumin (M_r 67 000); ovalbumin (M_r 43 000); carbonic anhydrase (M_r 30 000); trypsin (M_r 20 100); α-lactalbumin (M_r 14 400). Coomassie brilliant blue was used to stain the resolved proteins.

~28 000. Our results do not allow us to conclude that the dimer was composed of identical subunits because on some gels the monomer form appeared as two closely migrating bands. Thrombolectin did not appear to be a glycoprotein because it gave negative reactions to the Schiff's reagent following treatment with periodate. The average specific activity for all our assays (pH 7.4) of the purified thrombolectin was 86.8×10^6 (S. A. is the hemagglutination titer/mg protein). Ethylenedinitrilo–tetraacetic acid (1 mM) abolished thrombolectin's hemagglutination activity and this inhibition was prevented by calcium. Over pH 6.0–9.0, thrombolectin had maximum hemagglutination activtiy at pH 6.6. Dithiothreitol (2 mM) inhibited ⩾87% of the lectin activity. Hemagglutination was inhibited with decreasing efficiency by the following compounds: TDG; lactose; methyl β-D-galactoside; D-galactose; and methyl α-D-galactoside (see table 2). Inhibition of 50% of the maximum hemagglutination activity was caused by 0.3 mM

Table 2
Saccharide inhibition of thrombolectin hemagglutination activity

Saccharide	Concentration that inhibits 50%[a] of maximum activity (mM)
Thiodigalactoside	0.3
Lactose	0.8
Methyl β-D-galactoside	2.0
D-Galactose	2.0
Methyl α-D-galactoside	6.0
Maltose	>15
Sucrose	>15
D-Glucose	>15
D-Mannose	>15
D-Fructose	>15

[a] D-Galactosamine, D-glucosamine, D-mannosamine, *N*-acetyl glucosamine, methyl α-D-mannoside, arginine, glutamine, and lysine also failed to inhibit at 15 mM final conc.

TDG, 0.8 mM lactose, 2 mM methyl β-D-galactoside, 2 mM D-galactose, or 6 mM methyl α-D-galactoside (table 2). Thrombolectin was stable during both storage at −12°C and overnight incubation at room temperature.

4. Discussion

These results demonstrate that the venom of *Bothrops atrox* contains thrombolectin. The hemagglutination activity of thrombolectin was inhibited by TDG, lactose, and methyl β-D-galactoside. Therefore, thrombolectin has a similar, though not identical, saccharide specificity as a class of lectins (electrolectins [13] or galactolectins [4]) originally isolated from the electric organ of the electric eel [13] and isolated or identified in a variety of rat tissues [13], chick embryonic pectoral and thigh muscles [14–16], brain and liver [17], adult chicken liver, pancreas and intestine [18], rat myoblasts in tissue culture [19], chicken heart and calf spleen, thymus, liver, heart [20], and lungs [6]. Characterization of galactolectins purified from these tissues [12–14,17,20] demonstrated that they also were dimers but differ, in those cases tested, from thrombolectin in that they were not calcium-dependent, required thiol groups for activity, and were inhibited more extensively by methyl α-D-galactoside than by methyl β-D-galactoside.

The physiological role(s) of galactolectins is unknown. However, evidence obtained using different

systems supports the contention that the function of the β-galactoside-binding lectin present in myoblasts is prerequisite for fusion of the myoblasts [12,19, 21,22]. The role of this lectin in myotube formation is not understood. Because lectins with the same specificity occur in a variety of tissues it has been suggested that the same lectin may have a different function in different tissues [18], or that the lectin may have a common function which is expressed at different times during development [4]. Despite these speculations, we have no understanding of the function of thrombolectin in the *Bothrops atrox* venom. However, the fact that thrombolectin is present in venom of *Bothrops atrox* suggests that binding of thrombolectin to receptors of galactolectins in animals bitten by *Bothrops atrox* may contribute to the death of the injured animal. This raises the possibility that the function(s) of galactolectins and/or their receptors is required for the viability of a variety of animals.

Although, to our knowledge, this is the first report of a lectin isolated from a snake venom, there are other observations reported in the literature which may be explicable in these terms. A glycoprotein was isolated from the venom of the pit viper *Trimeresurus okinavensis* [23]. This glycoprotein lacks caseinolytic, esterolytic and coagulant activities, yet it causes the aggregation of blood platelets. Similar activity was identified in the venoms of *Crotalus terrificus* and *Trimeresurus purpureomaculata* [24]. A platelet aggregating agent was identified in the venom of *Crotalus horridus horridus* [25]. Also, a coagglutinin for von Willebrand factor was identified in *Bothrops* venom [26]. As thrombolectin causes platelet aggregation (T. K. G., D. C. W., submitted) in each or some of the above examples, a lectin may be one of the platelet aggregating components present in the venoms. Further work is required to clarify this issue.

Acknowledgement

This research was supported by project grant HL 23010 from the US Public Health Service.

References

[1] Stocker, K. and Barlow, G. H. (1976) Methods Enzymol. 45, 214–222.
[2] Kirby, E. P., Niewiarowski, S., Stocker, K., Kettner, C., Shaw, E. and Brudzynski, T. M. (1979) Biochemistry 18, 3564–3570.
[3] Niewiarowski, S., Kirby, E. P., Brudzynski, T. M. and Stocker, K. (1979) Biochemistry 18, 3570–3577.
[4] Phillips, D. R. and Gartner, T. K. (1980) in: Bacterial Adherence (Beachey, E. H. ed) pp. 399–438, Chapman and Hall, London.
[5] Gartner, T. K., Williams, D. C. and Phillips, D. R. (1977) Biochem. Biophys. Res. Commun. 79, 592–599.
[6] De Waard, A., Hickman, S. and Kornfeld, S. (1976) J. Biol. Chem. 251, 7581–7587.
[7] Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059–3065.
[8] Phillips, D. R. and Agin, P. P. (1977) J. Biol. Chem. 252, 2121–2126.
[9] Clarke, J. T. (1964) Ann. NY Acad. Sci. 121, 428–436.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1955) J. Biol. Chem. 193, 265–275.
[11] Gartner, T. K., Williams, D. C., Minion, F. C. and Phillips, D. R. (1978) Science 200, 1281–1283.
[12] Den, H. and Malinzak, D. A. (1977) J. Biol. Chem. 252, 5444–5448.
[13] Teichberg, V. I., Silman, I., Beitsch, D. D. and Resheff, G. (1975) Proc. Natl. Acad. Sci. USA 72, 1383–1387.
[14] Nowak, T. P., Kobiler, D., Roel, L. E. and Barondes, S. H. (1977) J. Biol. Chem. 252, 6026–6030.
[15] Nowak, T. P., Haywood, P. L. and Barondes, S. H. (1976) Biochem. Biophys. Res. Commun. 68, 650–657.
[16] Den, H., Malinzak, D. A. and Rosenberg, A. (1976) Biochem. Biophys. Res. Commun. 69, 621–627.
[17] Kobiler, D., Beyer, E. C. and Barondes, S. H. (1978) Dev. Biol. 64, 265–272.
[18] Beyer, E. C., Tokuyasu, K. T. and Barondes, S. H. (1979) J. Cell Biol. 82, 565–571.
[19] Gartner, T. K. and Podleski, R. T. (1975) Biochem. Biophys. Res. Commun. 67, 972–978.
[20] Briles, E. B., Gregory, W., Fletcher, P. and Kornfeld, S. (1979) J. Cell Biol. 81, 528–537.
[21] Gartner, T. K. and Podleski, R. T. (1976) Biochem. Biophys. Res. Commun. 70, 1142–1149.
[22] MacBride, R. G. and Przybylski, R. J. (1980) J. Cell Biol. in press.
[23] Davey, M. G. and Esnouf, M. P. (1969) Biochem. J. 111, 733–743.
[24] Davey, M. G. and Lüscher, E. F. (1967) Nature 216, 857–858.
[25] Schmaier, A., Colman, R. W. and Claypool, W. (1978) Circulation 58 (II), 835.
[26] Read, M. S., Shermer, R. W. and Brinkhous, K. M. (1978) Proc. Natl. Acad. Sci. USA 75, 4514–4518.