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Theromin, a Novel Leech Thrombin Inhibitor*

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We purified the most potent thrombin inhibitor described to date from the rhynchobdellid leech *Theromyzon tessulatum*. Designated theromin, it was purified to apparent homogeneity by gel permeation and anion exchange chromatography followed by two reverse-phase steps of high performance liquid chromatography. The primary sequence of theromin (a homodimer of 67 amino acid residues including 16 cysteine residues) was determined by a combination of reduction and s-β-pyridylethylation, Edman degradation, trypsin enzymatic digestion, and matrix-assisted laser desorption mass spectrometry measurement. Theromin exhibits no sequence homology with any other thrombin inhibitors. Furthermore, theromin significantly diminishes, in a dose-dependent manner, the level of human granulocyte and monocyte activation induced by lipopolysaccharides. In summary, this potent thrombin inhibitor promises to have high biomedical significance.

To understand pathological phenomena associated with a high level of immunocyte activity, scientists have used hematophagous animals as models to discover new anticoagulants. Such animals, naturally dependent on a diet of fresh blood, have evolved a variety of mechanisms that interfere with the coagulation process of the blood donor.

Normally a nociceptive stress due to being bitten will lead to an inflammatory response with leukocyte accumulation. Leeches, for example, avoid this in their own intestines by releasing (1) blood-degrading enzymes from leukocytes during the months used to digest the blood meal. Thus, the challenge for leeches while feeding is to block the victim's peripheral nociception, local inflammation, and coagulation (1–5). In this regard, opiate molecules (1, 2), cannabinoids (3), potent antinociceptive molecules (4), a variety of coagulation inhibitors (5), and immunomodulators (5) have been isolated from leeches.

In these animals, two groups of serine protease inhibitors have been found. One is related to the specific inhibitors of proteases that interfere with the activation of blood clotting processes. These include thrombin inhibitors such as hirudin (6) and haemadin (7) and coagulation factor Xa inhibitors such as antistasin (8) and ghilanten (9). The second group of inhibitors acts on the extracellular matrix; these include, for example, bdellins (10), eglins (11), gelin (12), guamerins (13, 14), and hirustasin (15).

Among the different anticoagulant molecules, hirudin was first described in 1884 by Haycraft (16), who discovered it in salivary glands of the leech *Hirudo medicinalis*. In 1927, Shionoya (17) investigated its involvement as a potent antithrombosis drug. Its definitive structure was determined in 1957 by Markwardt and Leberecht (18). Hirudin is a natural single-chain peptide of 65 residues with three intra-chain disulfide bridges and a sulfated tyrosine residue (19–22). Its N-terminal part is globular and very tightly clustered because of the presence of the three disulfide bridges. By contrast, its C-terminal part is rather light, with numerous negatively charged amino acid residues. Over 100 years after its discovery, its cDNA was cloned, and the recombinant hirudin has been obtained in *Escherichia coli* (23), yeast *Saccharomyces cerevisiae* (24), and recently *Acremonium chrysogenum* (24). The commercial development, pre-clinical evaluation, and introduction into clinical trials of the recombinant and analog forms of hirudin were achieved only 10 years ago (25, 26).

Compared with heparin, hirudin is a strict thrombin inhibitor that does not need a co-factor for its activity. Hirudin makes an irreversible tight bond to thrombin. The complex formed by hirudin and thrombin is very close and extends to a large area around the active site (27). The last 10 amino acid residues of the C-terminal region (residues 55–65) react with the anionic site of thrombin, an important region for fibrinogen binding. Residues 1–48 of the N-terminal part are also important for the action of hirudin on thrombin (25, 28), because they interact with the catalytic site. In this context, amino acid residues 46–48 are extremely important for the link between hirudin and thrombin. These types of interactions explain why hirudin only binds to thrombin and not to other serine proteases.

Up to now, only two other less potent thrombin inhibitors, haemadin (7) and bufrudin (30), have been isolated, both from jawed leeches. Here we present a new highly potent thrombin inhibitor isolated from a gut leech, *Theromyzon tessulatum*.

EXPERIMENTAL PROCEDURES

Leeches—Starved *T. tessulatum* leeches were maintained in our laboratory as described elsewhere in detail (31).

Materials—Chromogenic substrates S-2765, S-2238, and S-2586 were purchased from Kabi Diagnostica. High pressure liquid chromatography (HPLC)[1]-grade acetonitrile was obtained from J. T. Baker Inc.

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The amino acid sequence reported in this paper has been submitted to the Swiss Protein Database under Swiss-Prot accession no. P82354.

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[1] The abbreviations used are: HPLC, high pressure liquid chromatography; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; MALDI

Trifluoroacetic acid was from Pierce. Porcine pancreatic elastase (EC 3.4.21.36), chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), cathepsin G (EC 3.4.21.20), thrombin (EC 3.4.21.5), and chromogenic substrates (benzoyl-arginine *p*-nitroanilide, *N*-succinyl Ala-Ala-Ala *p*-nitroanilide, and *N*-succinyl Ala-Ala-Pro-Phe *p*-nitroanilide) were obtained from Sigma. Molecular weight calibration markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Amersham Pharmacia Biotech. All other reagents were of analytical grade.

Isolation of Theromin—After anesthetizing the animals in 0.01% chloretone, the heads of *T. tessulatum* individuals were excised, frozen immediately in liquid nitrogen, and stored at −70 °C. Four-gram aliquots were placed in 25 ml of Tris/NaCl buffer (20 mM Tris/HCl, 200 mM NaCl, pH 8.4) and homogenized at 4 °C with a polytron in Tris/NaCl buffer (five 15-s bursts on setting 9). After centrifugation (30 min at 12,000 × *g* on a Sigma 2K15 centrifuge at 4 °C), the pellet was extracted 6-fold with Tris/NaCl. Supernatants were combined and filtered on nitrocellulose membranes (0.45-μm pore size, Millipore). The extract was applied onto a fast performance liquid chromatographic column (Superdex G75, 16/60, Amersham Pharmacia Biotech) (equilibrated with Tris/NaCl) at a flow rate of 1 ml/min and eluted with the same buffer. The column effluent was monitored by light absorbance at 280 nm. All column fractions (1 ml) were assayed for protease inhibitor activity against thrombin. Pooled active fractions were concentrated 20-fold in a vacuum centrifuge (Savant) before being loaded onto a Mono Q fast performance liquid chromatography column (HR 5/5, Amersham Pharmacia Biotech) equilibrated in 20 mM Tris/HCl, pH 8.8. The column was washed with the same buffer and eluted with a discontinuous linear gradient of 0.2–1.5 M NaCl for 60 min at a flow rate of 1 ml/min. Fractions containing putative theromin were applied to a C8 Lichrosphere Rp100 column (125 × 4.6 mm, Merck) with a linear gradient of acetonitrile at 3.3%/min in water acidified with 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The active fractions were then purified on the same column with a shallower gradient (1%/min). All HPLC purifications were performed with a Beckman Gold HPLC system equipped with a photodiode array detector (Beckman 168).

To follow the theromin at each step of purification, chromogenic assays were performed. These were carried out at room temperature in 96-well microtiter plates. The color developed from the hydrolysis of peptide-nitroanilide substrates was monitored at 405 nm on a Dynatec MR-250 microtiter reader. The concentration of thrombin was given by Bradford's procedure (32) using γ globulin as standard and confirmed by amino acid analysis. Typically, the assay included 3 μM proteolytic enzyme in 20 mM Tris/HCl (pH 8.4), 0.2 M NaCl and an aliquot of selected column fractions in a total volume of 100 μl. After 15 min of incubation, the assay was started with the addition of the chromogenic substrate. The released nitroaniline was monitored photometrically at 405 nm for 3.5 min using a Dynatec MR-5000 photometer at room temperature. A control reaction, in the absence of theromin, was carried out under identical conditions. The reaction was stopped by the addition of 0.2 ml of 50% glacial acetic acid. One unit of protease inhibitory activity is defined as 1 μg of protease inhibited, and specific activity is defined as units/mg protease inhibited (33). Purification was performed by SDS-PAGE. Electrophoretic analysis of reduced and denatured proteins was performed in 10–25% SDS-polyacrylamide gradient gels in the presence of β-mercaptoethanol as described by Laemmli (34).

Theromin Characterization—Before microsequencing the theromin, the purity of the peptide was evaluated by capillary electrophoresis. Samples (2 nl) were injected under vacuum into a 270A-HT capillary electrophoresis system (Applied Biosystems) equipped with a fused silica capillary (length, 72 cm). Separation from anode to cathode was carried out in 20 mM citrate buffer (pH 2.5) with a voltage of 20 kV at 30 °C. Capillary effluent was monitored by UV absorption at 200 nm. Moreover, to perform Edman degradation, the peptide was reduced and alkylated. Ten microliters of inhibitor (50 pM) was dissolved in 40 μl of 0.5 M Tris/HCl (pH 7.5) and 2 mM EDTA containing 6 M guanidine hydrochloride, to which 2 μl of 2 M dithiothreitol were added in the dark. The sample was flushed with nitrogen and incubated at 45 °C for 1 h. Freshly distilled 4-vinylpyridine (2 μl) was added and incubated for 10 min at 45 °C in the dark. The pyridylethylated peptide was separated by reverse-phase HPLC before microsequencing. Automated Edman degradation of both native and pyridylethylated peptides and detection of phenylthiohydantoin derivatives were performed on a pulse-liquid automatic sequencer (Applied Biosystems 473A). Enzymatic digestion allowed for complete sequence determination. An aliquot of the *S*-β-pyridylethylated theromin was treated with trypsin (*N*-tosyl-L-phenylalanine chloromethyl ketone-treated, Takara Shuzo, Kyoto, Japan) at a ratio of 25% (w/w). The material was digested according to the manufacturer's directions (37 °C, 16 h). The reaction was stopped by acidification, and the peptide fragments were separated on an RP100 C18 column (250 × 4.6 mm, Merck) equilibrated with acidified water. Fractions were eluted with a linear gradient (0–80%) of acetonitrile in acidified water over 80 min at a flow rate of 1 ml/min. Emerging peaks were freeze-dried (Speed-Vac, Savant) before sequencing.

The mass of theromin was obtained by matrix assisted laser desorption/ionization mass spectrometry (MALDI MS). Aliquots of purified theromin (1 μl) were deposited on a thin water layer of α-cyano-4-hydroxycinnamic acid crystals made by fast evaporation of a saturated solution in acetone. The droplet was allowed to dry under a gentle vacuum before introduction into the mass spectrometer. MALDI MS measurement was performed in a Bruker BIFLEX™ spectrometer (Bruker, Bremen, Germany) operating in the positive linear mode. Ions were formed by a pulsed ultraviolet laser beam (nitrogen laser, λ = 337 nm). Mass spectra were obtained by averaging 50–100 laser shots. External mass calibration was provided by the $[M + H]^+$ ion of angiotensin II (1047.20 Da), ACTH (18–39) (2466.73 Da), and bovine insulin (5734.56 Da) and/or by using matrix peaks $[M + H]^+$ (190.05 Da) and $[2M + H]^+$ (379.09 Da).

Determination of Equilibrium Constants—To determine the specificity of theromin, its inhibitory activity toward different serine proteases was compared using established chromogenic assays. Equilibrium dissociation constants (K_i) for the complexes of theromin with individual proteases were determined essentially as described by Henderson (35) and as simplified by Bieth (36). Briefly, increasing amounts of theromin were incubated with a constant amount of protease in Tris/NaCl buffer for 30 min at 37 °C, and the remaining protease activity was measured by the addition of chromogenic substrate. The remaining protease activity was monitored by absorbance at 405 nm as described above. Graphical analysis yielded an apparent K_i using the equation $[I]/(1 - a) = K_{iapp}(1/a) + [P]$, where [I] and [P] are the initial concentrations of inhibitor and protease, respectively, and a is the remaining fractional protease activity (100% control activity equals fractional activity of 1.0). This is the remaining fractional protease activity (100% control activity equals a fractional activity of 1.0) (37).

Immunocyte Assay—Cells were separated by a standard Ficoll-Hypaque method as described elsewhere in detail (40, 41). They were washed three times in RPMI medium (RPMI, 25 mM Hepes, Grand Island Biological Co., New York, NY). Lipopolysaccharides (LPS) were obtained from Sigma. In this assay, immunocytes were analyzed for their ability to change shape in response to LPS. Both granulocytes and monocytes were diluted (406 ± 13 cells/100 μl) and exposed to LPS at a previously determined effective dose (1 unit/ml) on pre-coated (1% bovine serum albumin) glass slides. Changes in cell shape were monitored using computer-assisted microscopic image analysis (Image Analytics, Inc., Hauppauge, NY) over a 60-min period. After activation by LPS, the cells were co-incubated with aprotinin, a serine protease inhibitor, and tessulin (42), therin (43), or theromin.

Analysis of Cellular Activity—The analysis of immunocyte activation was determined as reported elsewhere (40). Briefly, cells with or without the various agents were analyzed using the computer-assisted microscopic image analysis. About 45–51 activated cells (amoeboid and exhibiting form factors below 0.5) were observed for each 400-μm viewing diameter, and four additional viewing diameters were observed for each slide to calculate a single mean value. The entire process was repeated three times, and the resulting mean of the mean (% immunocyte activation ± S.E.) was plotted against time. The variation for individual readings was between 5 and 9%.

Human immunocytes were analyzed every 15 min following initial exposure to the various agents. Changes in cellular conformation ranging from inactive rounded to active amoeboid were determined by measurements of cellular area and perimeter and were expressed mathematically using the shape factor formula ($4 \times p \times \text{area}/\text{perimeter}^2$). The proportion of activated cells was determined as described elsewhere (40). Activated cells not only change their conformation in response to a pharmacological stimulus, but also become mobile and capable of phagocytosis and secrete various signaling molecules (40, 41).

Statistics—Analysis was performed using the Student's *t* test to compare controls with LPS-stimulated cells and those stimulated with LPS in the presence of tessulin, therin, theromin, and/or aprotinin.

MS, matrix assisted laser desorption/ionization mass spectrometry; LPS, lipopolysaccharides.

TABLE I
Purification of theromin

Four grams of leech extract was used as the starting material. One inhibition unit (IU) was defined as the amount (μg) of thrombin inhibited at 100% (see "Experimental Procedures").

Step	Total protein	Total activity	Specific activity	Recovery	Purification
	mg	*IU*	*IU/mg*	*%*	*-fold*
Leech extract	400	1200	3	100	1
Superdex G75	40	1000	25	83	8
Mono Q fast protein liquid chromotography	1.7	425	250	35	83
Reverse phase HPLC2	0.006	105	17582	9	5860

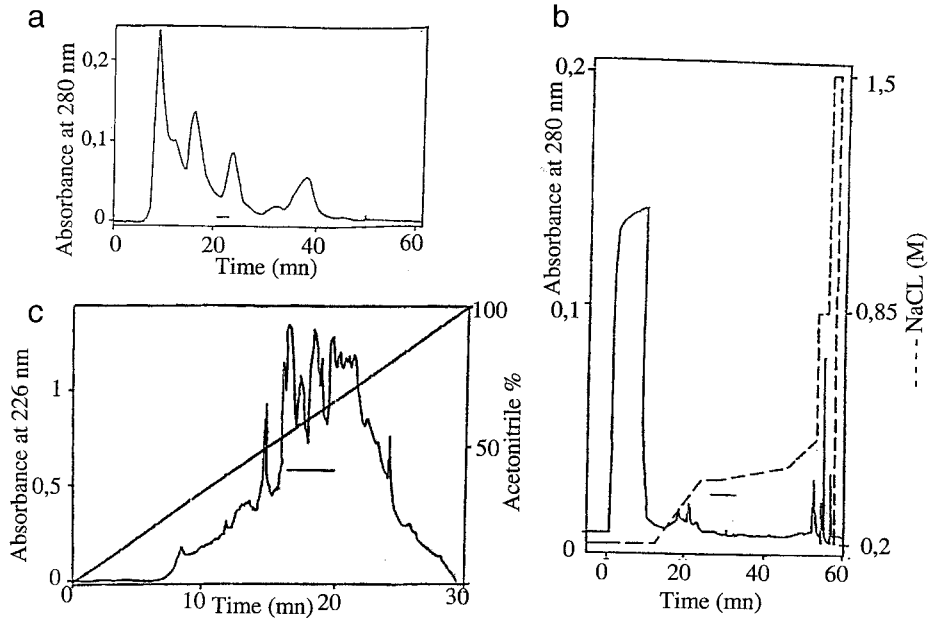

FIG. 1. **Elution profiles of theromin following purification steps.** *a*, elution profile on G75 column; *b*, elution profile on MonoQ column; *c*, elution profile on reverse phase HPLC. The *horizontal bars* indicate the active fractions.

Each experimental sample was compared with its own control to reduce variability due to spontaneous activation.

RESULTS

Purification of Theromin—Proteins extracted from the head preparations of *T. tessulatum* displayed thrombin-inhibiting activities (Table I). A single zone exhibiting thrombin inhibition activity was detected at a retention time between 21 and 23 min after gel permeation chromatography (Fig. 1*a*). This corresponded to proteins with a molecular mass less than 20 kDa (Fig. 2, *lane b*). This material was then purified by anion exchange chromatography as described above. The identified material eluted from the column at a NaCl concentration of 0.38–0.4 M (Fig. 1*b*). SDS-PAGE controls revealed several protein bands at a molecular mass of less than 20 kDa (Fig. 2, *lane c*). The peptide was finally purified by two successive reverse-phase HPLC procedures. Several peaks eluted from the column at retention times of 15–18 min, corresponding to 53.28–59.94% of acetonitrile in the first HPLC procedure (Fig. 1*c*). In the second HPLC procedure, a single peak exhibiting the activity was obtained at a retention time of 36.7 min, corresponding to 36.76% of acetonitrile (data not shown). After the final reverse-phase HPLC, the purity of the material was verified by capillary zone electrophoresis and SDS-PAGE (Fig. 2, *lane d*). A single band at a molecular mass of about 15 kDa was stained with Coomassie Brilliant Blue, confirming its purity.

Biochemical Characterization—Before microsequencing, the purified peptide was reduced and *S*-β-pyridylethylated and then subjected to automated Edman degradation. The first 54 amino acid residues were obtained on 25 pM isolated theromin with a repetitive yield of 96%. These residues were ^{1}ECENTECPRACPGEYEFDEDGCNTCVCKGCDDAQCRCSSDANGCESFCTCNTRC^{54}.

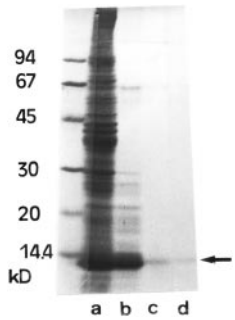

FIG. 2. **SDS-PAGE under reducing conditions of fractions containing theromin.** *Lane a*, crude extract; *lane b*, after Superdex G75 column separation; *lane c*, after MonoQ column separation; *lane d*, after the final step of purification on a C8 reverse phase column. The *arrow* indicates the position of theromin. *kD*, kilodalton.

Trypsin digestion was then performed followed by a reverse-phase separation (Fig. 3*a*). Each peak was then measured by MALDI MS before being subjected to automated Edman microsequencing (Table II). This strategy allowed for the determination of the complete sequence of the thrombin inhibitor designated theromin (Fig. 3*b*). The sequence was ^{1}ECENTECPRACPGEYEFDEDGCNTCVCKGC DDAQCRCSSDANGCESFCTCNTRCSAADECNPRCTCK^{67}.

This inhibitor is composed of 67 amino acid residues with a calculated mass of 7214.69 Da. By contrast, the molecular mass measured by MALDI MS of the native molecule had a mass of 14,491 Da, which is in agreement with that found by SDS-PAGE. However, after reduction and *S*-β-pyridylethylation of the molecule, the mass measured shifted to a lower value of

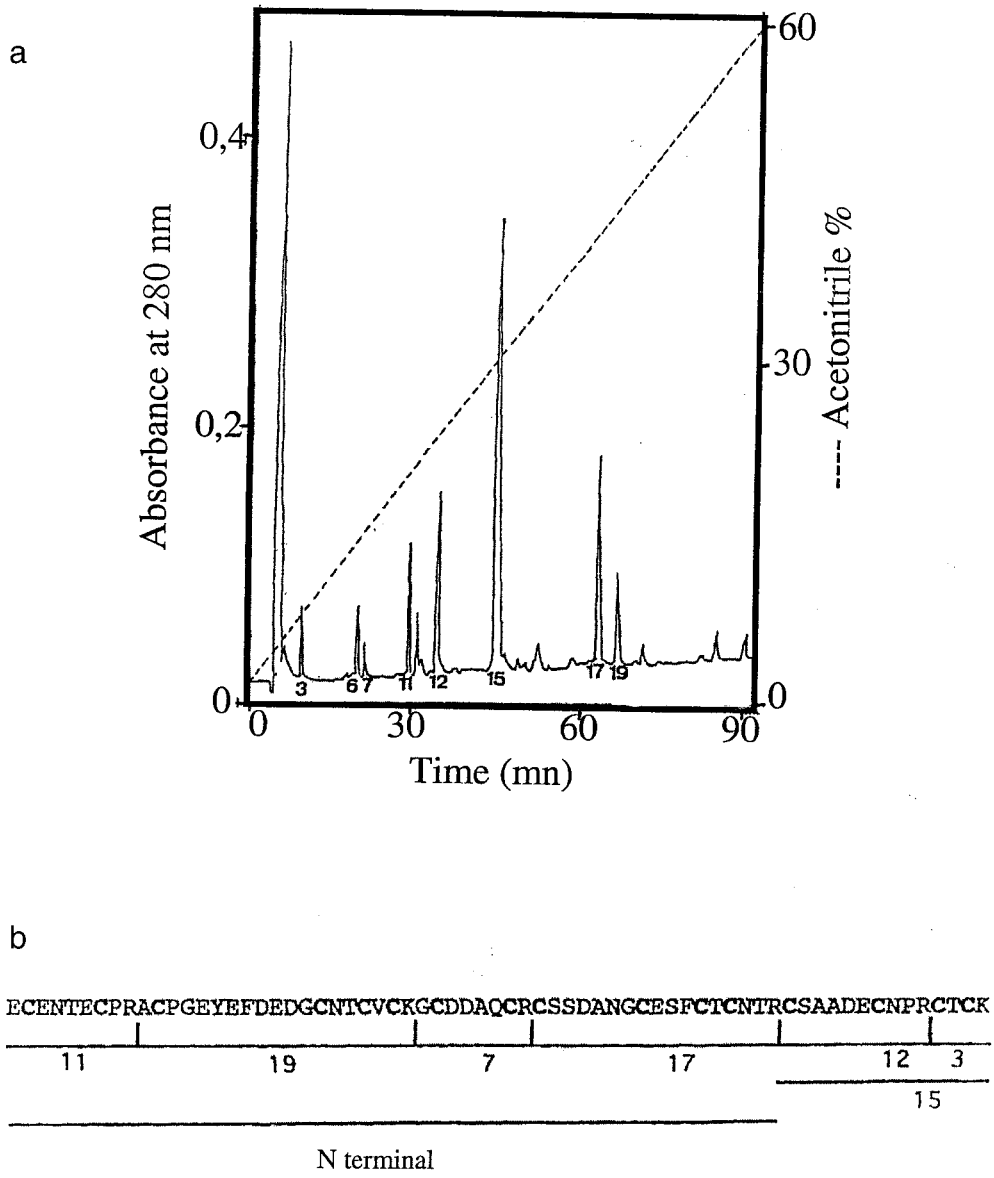

FIG. 3. **Amino acid sequence of theromin.** The sequence (67 amino acid residues) results from the analysis of the *S*-β-pyridylethylated theromin that provided good yields for 54 residues on a pulse-liquid automatic sequencer (Beckman, model LF 3200). Trypsin digestion followed by fractionating digested peptides on reverse phase HPLC with a Lichrosphere C8 column (*a*) was used to obtain the complete sequence (*b*).

8911 Da (data not shown). This measured mass is in agreement with the monoisotopic molecular mass of theromin calculated from the amino acid sequence with 16 cysteines (7214.69 Da). Taken together, the data demonstrate that theromin is a homodimer of about 14.5 kDa.

Sequence comparisons between theromin and all other known thrombin inhibitors like hirudin (2) and haemadin (6) revealed no sequence homology, confirming that theromin is a novel thrombin inhibitor. Similarly, this molecule exhibits no significant sequence identity with leech inhibitors of the antistasin-type family, namely antistasin (3), ghilanten (4), hirustasin (10), and guamerins (8, 9). Moreover, theromin did not express the leech antihemostatic protein (44) consensus motif (Cys-X_{6-12}-Cys-*X*-Cys-X_{3-6}-Cys-X_{3-6}-CysX_{8-14}) previously found in hirudin (2), decorsin (45), and antistasin (3), further confirming its novelty.

Theromin Biological Activity—To compare theromin with other serine proteases already isolated from leeches (5), we checked its specificity. Theromin, like hirudin, has no activity toward trypsin, chymotrypsin, elastase, cathepsin G, plasmin, or factor Xa (EC 3.4.21.6). By contrast, under the same conditions as hirudin, theromin demonstrated activity on the S-2238 chromogenic substrate with approximately 17,500 inhibition units/mg of protein (Table I) and a K_i value of 12 ± 5 fM. This value is higher than that obtained with hirudin (21 fM) (6) and

TABLE II
Amino acid sequences and mass determinations of tryptic peptides of theromin

S-β-pyridylethylated peptides generated by trypsin digestion were resolved by reverse phase HPLC and subjected to Edman degradation and MALDI-time of flight MS measurement.

Peak Number	*m/z*	Sequences
3	691.57	CTCK
6	906.79	CTCNTR
7	1078	GCDAQCR
11	1290.15	ECENTECPR
12	1275.14	CSAADECNPR
15	1920.7	CSAADECNPRCTCK
17	2217.92	CSSDANGCESFCTCTCNTR
19	2505.6	ACPGEYEFDEDCNTCVCK

TABLE III
Comparison of inhibitory constants (K_i) from different thrombin inhibitors isolated from leeches

Enzymes	Theromin	Hirudin	Hemadin
Chymotrypsin	ϕ[a]	ϕ	ϕ
Trypsin	ϕ	ϕ	ϕ
Cathepsin G	ϕ	ϕ	ϕ
Plasmin	ϕ	ϕ	ϕ
Urokinase	ϕ	ϕ	ϕ
Elastase	ϕ	ϕ	ϕ
Thrombin	12 fmol	21 fmol	100 fmol
Factor Xa	ϕ	ϕ	ϕ

[a] ϕ, no inhibition.

haemadin (100 fM) (7) (Table III). Theromin is a strict tight binding thrombin inhibitor. Reduced and *S*-β-pyridylethylated theromin had little thrombin activity, suggesting that dimerization is necessary to give the protein an active folded configuration for complete binding to thrombin. Nevertheless, the reduced molecule does display anti-thrombin activity, suggesting that each monomer possesses an active site. This could explain the high value of 12 fM for theromin (dimer) compared with 21 fM for hirudin (monomer). Each monomer thus acts in synergy to block thrombin.

Activity on Human Immunocytes—As a serine protease inhibitor, aprotinin is used clinically to reduce or prevent the adverse effects caused by serine proteases during surgery, including bleeding and the need for blood transfusions. We tried to ascertain if theromin can have similar effects (46). In addition to its anti-fibrinolytic activity, aprotinin also diminishes the diffuse inflammatory responses associated with major surgery (46). In these conditions, we examined theromin for its ability to diminish LPS-induced excitation of human leukocytes. These leukocytes (granulocytes and monocytes) were chosen for their ability to release numerous enzymes and signal molecules that require processing to liberate excitatory molecules such as opioid peptides that may participate in a further enhancement of immunocyte activation (40, 46).

As previously observed, once human immunocytes are separated and placed on a slide, they exhibit a low level of spontaneous activation characterized by an increase in the number of cells becoming amoeboid and moving (see References 4 and 40 and Fig. 4). In the presence of a previously demonstrated effective dose of LPS (1 unit/ml) (see References 41–43), the number of activated cells increased significantly ($p < 0.005$, Fig. 4). Interestingly, this response to LPS continued after it was washed off (Fig. 5), suggesting that a cascading process that serves to further activate the cells (4, 40) may have been initiated. The results (Figs. 4 and 5) demonstrate that preincubation of immunocytes with LPS followed by aprotinin significantly ($p < 0.005$) diminished the immunocyte excitation caused by LPS in a concentration-dependent manner. This suggests that enzymes were present that may have been processing released signaling molecules, leading to the higher level of activation in the absence of aprotinin ($p < 0.01$, Fig. 5). Furthermore, the level of activation that occurs in the presence of aprotinin is higher than that found in non-LPS-exposed cells (10.8 ± 3.4 compared with 21.1 ± 3.7 in 10^{-6} M aprotinin, $p < 0.05$). This last result also supports our hypothesis that the higher level of cellular activation seen in the presence of aprotinin is due to a cascade of signaling molecules after LPS stimulation (46).

In this regard, exposure to theromin resulted in a concentration-dependent inhibition of cell activation in response to LPS similar to that found for aprotinin (Fig. 5). Taken together, these results demonstrate that, after a primary stimulus is given, the cells are capable of enhancing their own level of stimulation by generating signaling molecules that must be processed. In this regard, theromin is able to lower the level of activation significantly.

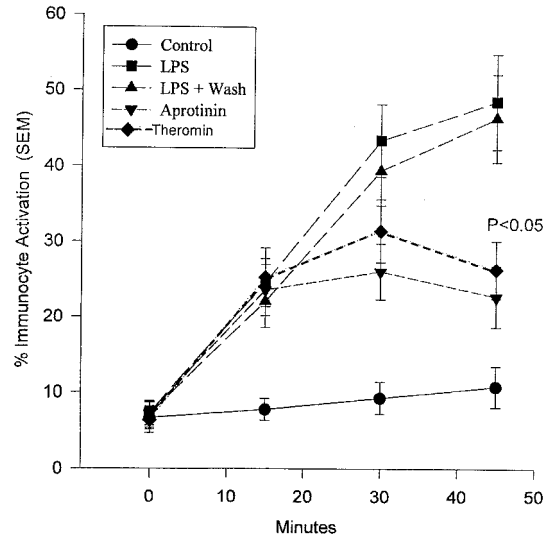

FIG. 4. **Demonstration of the dual nature of LPS activation of human monocytes.** LPS (1 unit/ml) stimulates monocytes in a manner that can be diminished by aprotinin (10^{-6} M) and theromin (10^{-6} M). The percentage activation is noted by computer-assisted microscopy, where the number of cells exhibiting form factors below 0.50 is compared with that of cells exhibiting this factor at 0.70 and above. Details of the assay are found in the text.

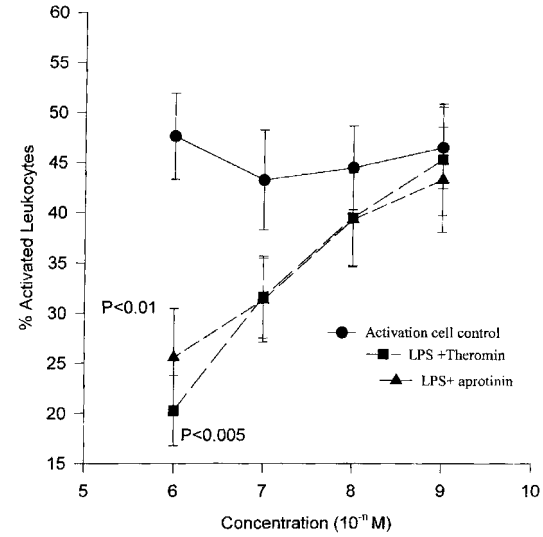

FIG. 5. **Concentration-dependant inhibition of LPS-activated human monocytes by aprotinin and theromin.** Details are in the text and the Fig. 4 legend.

DISCUSSION

Most of the polypeptides belonging to the family of thrombin inhibitors have been isolated from jawed leeches, such as the amphibian parasite leech *Hirudo medicinalis* (1), the mammalian parasite *Hirudinaria manillensis* (30), or the land-living *Hemadipsa sylvestris* (2). In the present study, we have for the first time isolated a specific tight binding thrombin inhibitor, theromin, from the gut leech *T. tessulatum*. This is a homodimer of 67 amino acid residues with 16 cysteines engaged in eight disulfide bridges. Compared with hirudin, a natural single-chain peptide of 65 residues with three intra-chain disulfide bridges and a sulfated tyrosine residue, both peptides are anionic and rich in cysteine residues (6). The hirudin N terminus is globular and very tight because of the presence of the three-disulfide bridges, and its C terminus is rather light

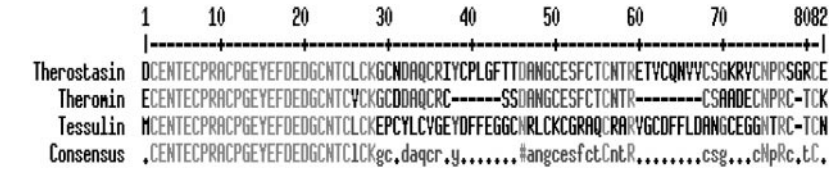

FIG. 6. **Sequence alignment between theromin, therostasin, and tessulin obtained using Multalin software.**

with numerous amino acid residues (33–36). In contrast, the N-terminal sequence of theromin is highly anionic, and its C-terminal part is very tight because of the 10 cysteine residues present there.

The hirudin N-terminal sequence is known to interact with the catalytic site of thrombin (6). In this context, amino acid residues 46–48 (PKP) are extremely important for the link between hirudin and thrombin (30). The same sequence has also been found in haemadin (2) and *H. manillensis* anti-thrombin peptides (30). These types of interactions explain why hirudin only binds to thrombin and not to other serine proteases. By contrast, theromin does not possess such a dramatic signature in its sequence. In fact, theromin has no significant sequence homology with any other animal thrombin inhibitor isolated so far. However, if theromin, which contains about 24% cysteine residues, is compared with potentially homologous inhibitors, low identity is observed with antistasin-type protease inhibitors and consists mainly of cysteine residues. However, considering the low level of general sequence identity between theromin and peptides of this family, it is difficult to consider theromin as a new member of the antistasin-type family. Additionally, these molecules differ in amino acid sequence and inhibitory activity (some of them in three-dimensional structures that share the same motif as that of leech antihemostatic protein) (43). Interestingly, the mechanisms of action and epitopes important for binding to their respective targets are distinct (5). However, theromin does not possess such a consensus motif.

By contrast, sequence comparisons were carried out for theromin with the four different serine protease inhibitors isolated from the leech *T. tessulatum*: cytin, therin, therostasin, and tessulin (5, 29, 41–43). This revealed that two of the four (*i.e.* therostasin and tessulin) have a high degree of sequence identity with theromin (70% and 52%, respectively) (Fig. 6). Furthermore, if theromin is aligned with these potentially homologous inhibitors, the three proteins show an identity from residues 2–28, with the exception of Val/Leu substitution at position 26 for theromin (Fig. 6). The rest of the sequences are less similar to each other. However, between therostasin and theromin another region is also well conserved; *i.e.* a consensus sequence has been found in residues 40–53 of theromin (^{40}DANGCESFCTCNTR^{53}). Interestingly, the putative active sites of the trypsin inhibitor tessulin (^{25}CLCKEPC^{31}) (42) and the factor Xa inhibitor therostasin (^{33}AQCRIYC^{39}) (29) are not conserved in the thrombin inhibitor theromin. Thus, the observed similarities could be the result of an evolutionary divergence from an ancestral gene, arising after gene duplication and able to generate several peptides acting toward specific substrates.

We can also add to the above *Theromyzon* molecules the three other thrombin inhibitors discovered in *Theromyzon* by Hamberger and collaborators (47). In fact, in 1994 the Merck Company deposited foreign patent applications regarding three thrombin inhibitors having masses of 3, 9, and 14 kDa. Interestingly, the N-terminal sequence of the 9-kDa inhibitor, EDDNPGPPRACPGE (47), shows homology with those of theromin (ECENTECPRACPGE), the factor Xa inhibitor therostasin (DCENTECPRACPGE) (29), and the trypsin inhibitor tessulin (MCENTECPRACPGE) (42). This 9-kDa thrombin inhibitor peptide possesses a pI of 4.9, a clotting time in a fibrinogen test of >600 s/5 μl, and a specific activity at the final step of purification of 25 IU for thrombin inhibition and 0.2 IU for factor Xa inhibition. In addition, clotting fibrinogen assays performed on different species of *Theromyzon* (*T. binannulatum*, *T. cooperi*, *T. garjaewi*, *T. maculosum*, and *T. sexoculatum*) confirm the presence of thrombin inhibitor(s) in these gut leeches (47). These data reveal that *Theromyzon* possesses different isoforms of thrombin inhibitor. However, the 9-kDa protease inhibitor possesses thrombin and factor Xa inhibitory activities and, when we compared its activity to the one we obtained for theromin, we observed that it is not a tight binding inhibitor for thrombin. We suspect that this molecule might be a more specific inhibitor of another protease.

Hirudin, at concentrations from 0.1 to 2 μM, induces vasodilatation of PGF2α pre-contracted ring segments of porcine pulmonary arteries with intact endothelium (46). We sought to determine whether theromin could release endothelium-derived nitric oxide (NO) as well (38). However, theromin, regardless of concentration, did not stimulate endothelial NO release from human saphenous vein endothelial cells (data not shown). The vein fragments were judged to be in good condition because 1 μM morphine released NO (33.6 $\pm$ 4.6 nM NO) as expected (38).

Moreover, we have previously demonstrated that lipopolysaccharides stimulate immunocytes in a process that exhibits a primary and secondary phase of stimulation (43). The LPS-stimulated secondary phase appears to require enzyme-processed secretory products released from immunocytes (40–43). Aprotinin, a serine protease inhibitor used in clinical situations, reduces or prevents the adverse effects caused by serine proteases during surgery. It inhibits bleeding and reduces the need for blood transfusions. In addition to its antifibrinolytic activity, aprotinin also diminishes the diffuse inflammatory response associated with major surgery (46). We previously demonstrated that this molecule significantly reduces the level of immunocyte activation, probably by inhibiting this "secondary" processing, thus releasing or freeing immunocyte excitatory peptides. In this context, theromin also diminishes this action and acts in conjunction with the other *Theromyzon* serine protease inhibitors therin (43) and tessulin (44).

Taken as a whole, the present study demonstrates that these relatively primitive organisms have devised highly specific mechanisms to parasitize their hosts. At the same time, these mechanisms serve to indicate the significance of various host processes as well as to shed light on their "vulnerable" steps. Indeed, these vulnerable points of "alteration" may well be used clinically to curtail pathological processes, such as the diffuse inflammatory responses associated with major surgery (5), where deemed appropriate.

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