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Isolation of a Novel Kunitz Family Protease Inhibitor in Association with *Tethya* Hemolysin from the Sponge *Tethya ingalli*

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Aqueous extracts from the New Zealand sponge *Tethya ingalli* (Hadromerida) displayed potent cytotoxicity in the NCI's 60-cell-line human tumor panel. Fractionation of the extract by ammonium sulfate precipitation, gel filtration, ultrafiltration, and both hydrophobic interaction and reversed-phase chromatography resulted in the isolation of two biologically active proteins. The first protein, *Tethya* protease inhibitor (TPI), which was purified to homogeneity, inhibited trypsin with an EC_{50} of 65 nM. TPI had a molecular mass of 11 431 Da, and an isoelectric point of 8.2. A partial *N*-terminal amino acid sequence determined for TPI showed significant homology with protease inhibitors of the Kunitz family. The second isolated protein displayed potent cytotoxicity, with pronounced selectivity for certain tumor cell lines (e.g., ovarian, renal, CNS, and breast). The latter protein, which had an apparent molecular weight of 21 kDa (SDS−PAGE), also lysed human red blood cells (EC_{50} of 39 nM) and was similar to a hemolysin previously isolated from the sponge *Tethya lycinurium*.

Sessile marine organisms have evolved many strategies to defend themselves from predators and to aid in their capture of prey. One such strategy is the production and sequestration of toxic compounds that can damage predators (e.g., sea anemone cytolysins)[1] or render prey incapable of escape (e.g., conotoxins).[2] Sponges, in particular, are known to produce potent cytotoxins (e.g., halitoxin,[3] swinholide A,[4] discorhabdin C[5]). Some of these compounds, such as spongistatin[6] and halichondrin B,[7] are being examined as potential antitumor drugs.

For the present study, an aqueous extract from the New Zealand sponge *Tethya ingalli* (Hadromerida), which displayed potent, selective cytotoxicity in the NCI's 60-cell-line human tumor panel, was chosen for bioassay-guided fractionation. Previous work on this genus had identified several primary[8] and secondary metabolites,[9] as well as a proteinaceous hemolysin.[10] We report here the isolation of two biologically active proteins from *T. ingalli*, one a potently cytotoxic hemolysin and the other a novel protease inhibitor with homology to the Kunitz family of serine protease inhibitors.

Results and Discussion

The cytotoxicity-guided fractionation of aqueous extracts of *T. ingalli* resulted in the purification of two biologically active proteins. *Tethya* protease inhibitor (TPI), was purified to homogeneity by a sequence of fractional ammonium sulfate precipitation, gel permeation chromatography, ultrafiltration, and C-18 reversed-phase chromatography (Figure 1, top). The protein was homogenous by SDS−PAGE (Figure 1, bottom). The second protein, *Tethya* hemolysin, was substantially purified by ammonium sulfate precipitation, gel permeation chromatography, ultrafiltration, and hydrophobic interaction chromatography. Analysis by SDS−PAGE indicated that contaminating protein bands were still present in the most highly purified fractions. It should be noted that residual *Tethya* hemolysin copurified with TPI and could be completely disassociated only by the C-18 reversed-phase HPLC purification step.

Analysis of partially purified protein extracts from *T. ingalli* indicated that the cytotoxicity displayed by *Tethya* hemolysin was stable throughout the pH range tested (pH 5−9) and that the protein was tolerant of temperatures from −20 °C to room temperature for 2 weeks without significant reduction of activity. Furthermore, the addition of 5 mM DTT did not significantly alter the activity of TPI but did decrease the activity and specificity of the hemolysin. Finally, the addition of 10 mM EDTA or 10% glycerol did not affect the activity of either protein.

TPI was found to have a molecular mass of 11 431 Da by MALDI-TOF mass spectroscopy. This molecular weight correlated well with both the native molecular weight of 12 kDa deduced from analytical gel filtration and the 11-kDa estimate derived from SDS-PAGE under reducing conditions, leading to the conclusion that TPI was a monomeric protein. TPI had a basic pI of 8.2 by native 2D electrophoresis and was not stained by periodic acid−Schiff reagent, indicating that the protein was not glycosylated. The results of amino acid analysis of TPI are shown in Table 1. After alkylation of the reduced Cys residues with vinyl pyridine, *N*-terminal amino acid sequencing of the intact protein resulted in identification of a 45-residue sequence of TPI (Figure 2). Repeated attempts to digest the protein with the

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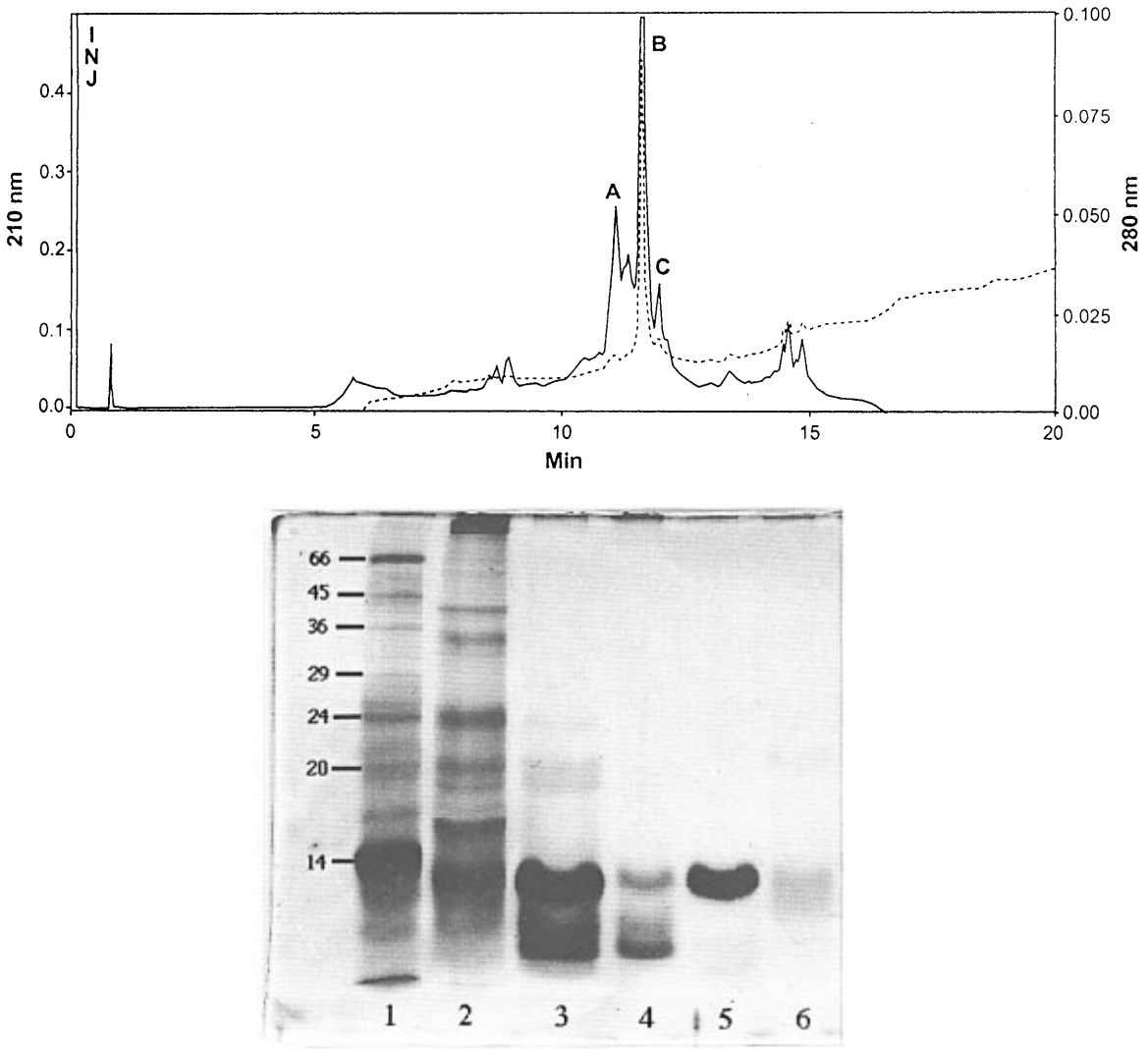

Figure 1. (Top) Chromatograph of the C-18 purification of TPI. A 200-μL sample of TPI, purified through the Sephadex G-100 gel filtration step, was injected onto a POROS II R/H C_{18} column (10 × 100 mm) and eluted as described in the Experimental Section. Absorbance was monitored at 210 (—) and 280 (- - -) nm. (Bottom) SDS-PAGE gel of the purification of TPI. Protein samples from the purification of TPI were subjected to electrophoresis on an 18% acrylamide gel at 30 mA constant current. The gel was stained with silver. Lane 1, molecular weight standards; lane 2, 35−55% ammonium sulfate precipitate; lane 3, active G-100 gel filtration column output; lane 4, C_{18} column fraction "A"; lane 5, C_{18} column fraction "B" containing purified, active TPI; C_{18} column fraction "C".

Table 1. Amino Acid Analysis of *Tethya* Protease Inhibitor

amino acid	molar %	residues	amino acid	molar %	residues
Asx	10.5	10	Ile	4.2	4
Thr	5.3	5	Leu	2.1	2
Ser	3.2	3	Tyr	7.4	7
Glx	9.5	9	Phe	2.1	2
Gly	14.7	15	His	3.2	3
Ala	9.5	9	Lys	6.3	6
Cys	6.3	6	Arg	4.2	4
Val	5.3	5	Pro	6.3	6
Met	1.1	1			

endoproteinases Lys-C and Glu-C were unsuccessful, yielding only trace amounts of cleavage peptides, even after denaturation with guanidine-HCl and a three-day incubation.

Tethya hemolysin was shown to have a molecular weight of 21 kDa by SDS−PAGE analysis under reducing conditions. Analytical gel permeation of the hemolysin also resulted in a determination of 21 kDa for the native molecular weight, thereby indicating that *Tethya* hemolysin was also monomeric. In 2D gel electrophoresis experiments, under native conditions, *Tethya* hemolysin displayed an isoelectric point of 6.2. Periodic acid−Schiff staining of the SDS−PAGE purified protein showed that the hemolysin was not glycosylated. All of the physical characteristics of *Tethya* hemolysin were very similar to those reported previously for a hemolysin isolated from the sponge *T. lycinurium*;[10] hence, the name of this protein has been conserved. Due to residual contaminating bands in the most highly purified fractions of *Tethya* hemolysin, neither amino acid analysis nor *N*-terminal sequencing was attempted.

Samples of *Tethya* hemolysin, purified through the G-3000 gel filtration step, displayed potent cytotoxicity in the NCI's 60-cell-line tumor panel (Figure 3), with a mean panel LC_{50} of 6.6 nM (0.14 μg/mL). Certain individual cell lines [e.g., OVCAR-3 (ovarian cancer), SNB-75 (CNS cancer), 786-0 (renal cancer), HS 578T (breast cancer)] were substantially more sensitive than the average; the overall range of differential sensitivity of the panel lines to the *Tethya* hemolysin was approximately 1000-fold. Visual inspection of OVCAR-3 cells immediately after the addition of *Tethya* hemolysin

	1	2	3	4 *	5	6
Tethya Protease Inh	.....alqD	FCnqPsdtGf	CRARfinYYY	NPrSNACEtF	tYGGacGNqN	NFKTKqECrl	aCigxxxxxx
Venom BPI III	.....rDrPk	FCyLPadpGr	ClAymPRfYY	NPASNkCEKF	IYGGCrGNAN	NFKTwdECrh	Tcvasgiqpr
Taicatoxin SPI	.....kDrPk	FChLPPkpGP	CRAaIPRfYY	NPhSkqCEKF	IYGGChGNAN	kFKTpdECny	Tclgvsl...
Cheonianin BPI	....qgDkrD	iCrLPPeqGP	CkgRIPRYfY	NPASrmCEsF	IYGGCkGNkN	NFKTKaECvr	aCRpPerpgv
Prot Inh 5 II	.......ing	dCeLPkvvGP	CRARfPRYYY	NssSkrCEKF	IYGGCgGNAN	NFhTleECek	vCgvrsVgR.
Colostrum Tr Inh	....fqtpPD	lCQLPqarGP	CkAallRYfY	NstSNACEpF	tYGGCqGNnN	NFeTtemCLr	iCepPqqtdk
Inter-a-trypsin Inh	dmsalfndse	aCkaaPetGP	CfgfIqgffY	NstSmrCElF	tYGGCLGNqN	NFvTvrECLq	rCRteaVCRL
Bikunin	litgtlkkeD	sCQLnyseGP	ClgmqeRYYY	NgASmACEtF	qYGGCLGNgN	NFisekdCLq	TCRtiaaCnL
Consensus	----------	-C------G-	C-^----^^Y	N--S--CE-F	-YGG*-GN-N	-F-^---C--	-C--------
		@@ active site					

Figure 2. *N*-terminal sequence of TPI and homology with Kunitz family protease inhibitors. *N*-terminal sequence of TPI was determined by automated Edman degradation. Sequence homology was determined using the GCG package (Genetics Computer Group, Madison, WI).

showed that the cell membranes of treated cells were lysed within minutes after the addition of the protein.

Partially purified G-100 fractions from *T. ingalli* displayed hemolytic activity against human erythrocytes at concentrations as low as 200 ng/mL. Analysis of more highly purified samples showed that only the early-eluting protein peak from the G-100 column was hemolytic. Experiments using hypotonically lysed erythrocyte membranes were then performed to examine if the hemolytic component of the protein sample was solely responsible for its cytotoxic activity. The results clearly demonstrated that, after preincubation with erythrocyte membranes, cytotoxic samples of Tethya hemolysin lost their cytotoxic activity. Furthermore, SDS−PAGE analysis of the supernatants from the erythrocyte membrane-binding experiment showed that a single protein band, corresponding to a molecular weight of 21 kDa, was absent from the supernatants (data not shown), thus indicating that this protein was both binding to the erythrocyte membranes and responsible for the cytotoxicity exhibited by this sample. One additional study was performed to compare the cytotoxicity and hemolytic activity of *Tethya* hemolysin after a 1-h incubation with either OVCAR-3 ovarian carcinoma cells (sensitive to the hemolysin), A-549 non-small-cell lung carcinoma cells (resistant to the hemolysin), or human type-O erythrocytes. The results (Figure 4) indicate that *Tethya* hemolysin destroys sensitive ovarian cancer cells ($IC_{50} = 0.16$ μg/mL), but not resistant lung cancer cells ($IC_{50} = 3.3$ μg/mL), more effectively than it lyses erythrocytes ($EC_{50} = 0.83$ μg/mL).

Experiments conducted with TPI showed that this protein did not lyse human erythrocytes at concentrations as high as 125 μg/mL. In addition, no diminution of protease inhibition was seen after the incubation of TPI samples with erythrocyte membranes.

Following the *N*-terminal amino acid sequence analysis of TPI, which indicated that the protein had significant homology to Kunitz family protease inhibitors, assays were conducted to determine its ability to inhibit trypsin. The results of these assays, as anticipated, showed that TPI was indeed a trypsin inhibitor, with potency ($EC_{50} = 0.74$ μg/mL) in the same range as the standard inhibitors aprotinin ($EC_{50} = 0.15$ μg/mL) and soybean trypsin inhibitor ($EC_{50} = 0.48$ μg/mL) (Figure 5).

Analysis of the 45 residue *N*-terminal amino acid sequence of TPI using the GCG program determined that strong homolgy (>50%) exists between TPI and several Kunitz-type protease inhibitors from the venoms of the snake locks sea anemone (protease inhibitor 5 II),[11] the Australian taipan (taicatoxin),[12] and the western sand viper (venom BPI III),[13] as well as to Chelonianin BPI (sea turtle)[14] and Bikunin (mouse),[15] colostrum trypsin inhibitor (bovine)[16] and inter-α-trypsin inhibitor (plaice)[17] (Figure 2). The presence of five cysteine residues at positions 6, 15, 31, 52, and 56 of TPI exactly matches the pattern found in the Kunitz family protease inhibitors, while the sixth cysteine residue, at position 40, is shifted one amino acid away from the consensus site in the homologous series. The putative active site of the Kunitz family protease inhibitors is also present in TPI at residues 15−17. Of particular importance is the presence of the arginine residue at position 16, as an arginine at this position is reported to confer specificity for the inhibition of trypsin-like proteases.[18] The isolation of TPI from the sponge *T. ingalli* and its identification as a Kunitz family protease inhibitor represent the most phylogenetically primitive source of this well-studied protein family to date. Previous work[19] has detailed the evolutionary development of the Kunitz-type trypsin inhibitor going back to the sea anemone (Coelenterata/Cnidaria). The isolation and identification of TPI extend the evolutionary history of Kunitz-type inhibitors many million years further back in time to the Porifera.

The co-occurrence of a protease inhibitor and a proteinaceous hemolysin has previously been reported in sea anemones and may confer some advantage against predation by fish.[20] Whether this same mechanism of protection, whereby a proteinaceous toxin is protected against enzymatic inactivation by association with a protease inhibitor, is utilized in *T. ingalli* will require additional investigation.

Experimental Section

General Experimental Procedures. All chemical reagents were purchased from Sigma (St. Louis, MO), electrophoresis standards were purchased from BioRad (Melville, NY), and proteases and substrates were purchased from either Sigma or Boehringer Mannheim (Germany). Protein content was determined by colorimetric assay using the BioRad protein assay system with bovine gamma globulin as the standard according to the procedures of Bradford.[21]

SDS−PAGE was carried out using 18% polyacrylamide resolving gels, 4% polyacrylamide stacking gels, and standard discontinuous buffer systems according to Laemmli[22] on a BioRad Mini Protean II apparatus.

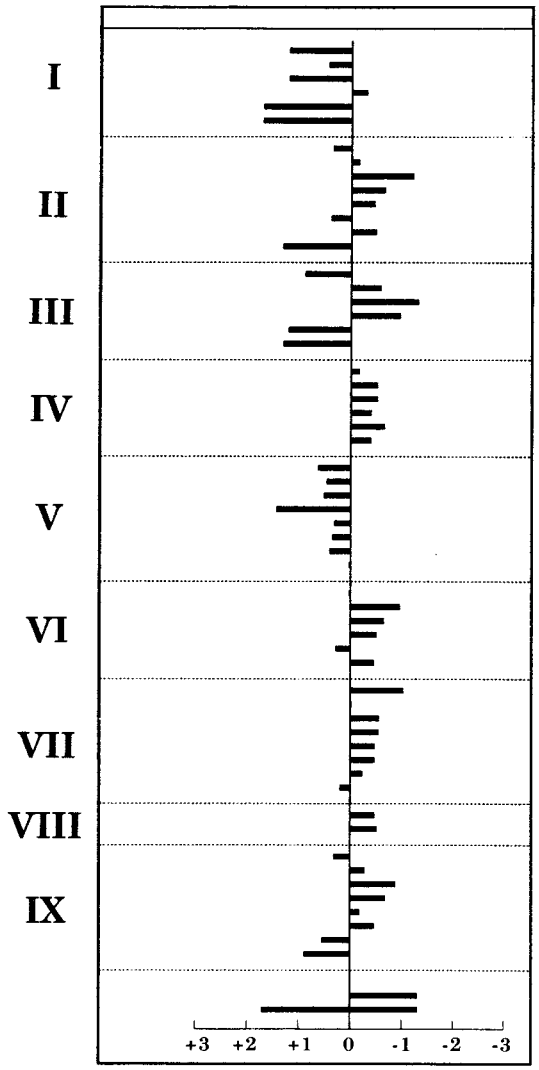

Figure 3. 60-cell data on *Tethya* hemolysin. LC_{50} mean bar graph (12) from screening of *Tethya* hemolysin in the NCI human tumor cell line panel. The tumor subpanels are identified as follows: I (leukemia); II (lung, non-small cell); III (colon); IV (CNS); V (melanoma); VI (ovarian); VII (renal); VIII (prostate); IX (breast). The mean $\log_{10}$ LC_{50} values (μg/mL), computed from triplicate screening of *Tethya* hemolysin, were as follows for the individual cell lines and corresponding subpanels (I−IX): [I] CCRF−CEM (0.36), HL-60 TB(−0.41), K-562 (0.36), MOLT-4 (−1.16), RPMI-8226 (0.85), SR (0.86); [II] A549/ATCC (−0.51), EKVX (−1.01), HOP-62 (−2.07), HOP-92 (−1.52), NCI-H226 (−1.31), NCI-H23 (−0.47), NCI-H322M (−1.35), NCI-H522 (0.48); [III] COLO 205 (0.04), HCT-116 (−1.43), HCT-15 (−2.17), HT29 (−1.82), KM12 (0.36), SW-620 (0.46); [IV] SF-268 (−1.02), SF-295 (−1.38), SF-539 (−1.38), SNB-19 (−1.26), SNB-75 (−1.52), U251 (−1.26); [V] LOX IMVI (−0.22), MALME-3M (−0.39), M14 (−0.34), SK-MEL-2 (0.59), SK-MEL-28 (−0.54), SK-MEL-5 (−0.51), UACC-257 (−0.44), UACC-62 (−0.82); [VI] IGROV1 (−0.82), OVCAR-3 (−0.82), OVCAR-4 (−1.51), OVCAR-5 (−1.37), OVCAR-8 (−0.57), SK-OV-3 (−1.33); [VII] 786-0 (−1.89), A498 (−0.89), ACHN (−1.42), CAKI-1 (−1.41), RXF-393 (−1.34), SN12C (−1.34), TK-10 (−1.10), UO-31 (−0.66); [VIII] PC-3 (−1.34), DU-145 (−1.38); [IX] MCF7 (−0.54), MCF/ADR-RES (−1.14), MDA-MB-231/ATCC (−1.74), HS 578T (−1.55), MDA-MB-435 (−1.05), MDA-N (−1.34), BT-549 (−0.31), T-47D (0.04). The $\log_{10}$ concentration (μg/mL) used as the reference point (centerline location) corresponds to 0.14 μg/mL, or 6.6 nM. Bars projecting to the right of the centerline represent cell lines that are proportionately more sensitive to the hemolysin, whereas bars projecting to the left of the centerline represent those that are proportionately less sensitive.

The gels (50 × 80 × 1 mm) were subjected to electrophoresis at a constant current of 30 mA/gel at room temperature with chilled electrode buffer. Native 2D gel electrophoresis was carried out using a nonurea 5% polyacrylamide tube gel system on a BioRad Mini Protean II apparatus. Protein samples were focused at 4 °C in a gradient from pH 3.5−9.5 using Sigma ampholines and the following voltage conditions: 500 V for 15 min followed by 750 V for 280 min. The isoelectric points of the proteins were determined by comparison to commercially available standard proteins (IEF Mix II, Sigma) run simultaneously. SDS−PAGE gels were stained for protein using either Coomassie brilliant blue or a modified Merrill silver stain procedure.[23] Gels were stained for glycoproteins by a periodic acid−Schiff stain (glycoprotein detection kit, Sigma).

Amino acid analysis was done using a Beckman model 6300 amino acid analyzer according to manufacturer protocols. *N*-terminal amino acid sequencing was performed on an Applied Biosystems model 477A sequencer according to manufacturer protocols, and sequence homology was searched using GCG Package, Version 8 (Genetics Computer Group, 575 Science Drive, Madison, WI 53711). Matrix-assisted laser desorption ionization−time of flight (MALDI-TOF) mass spectroscopy was done using a Kratos Kompact Maldi III instrument (Shimadzu) operated in a linear mode using sinapinic acid as a matrix and trypsin as an external standard.

Sponge Collection and Identification. *Tethya ingalli* (order Hadromerida, phylum Porifera) was collected by M. H. G. Munro on February 10, 1987, at a depth of 20 m in Leigh Cove, Maori Island, New Zealand by scuba, voucher no. D-Q66D0064-Z, stored at Smithsonian Institute Sorting Center, Suitland, MD, and identified by C. J. Battershill.

Preparation of Sponge Material. Sponge material was frozen immediately after collection and stored at −20 °C until extraction. The frozen sponge material was ground with solid CO_2 pellets in a mechanical grinder, and the CO_2 was allowed to sublime at −20 °C. The ground material was then thawed and brought up in DDH_2O, and the slurry was stirred for 4 h at 4 °C. The mixture was centrifuged using a basket centrifuge, and the filtrate was lyophilized to provide the aqueous extract.

60-Cell and 2-Cell Assay of Sponge Extracts. Analysis of cytotoxicity was by the NCI's in vitro 60-cell solid-tumor screening panel as reported previously.[24,25] Fractionation of the active proteins was also monitored by a 2-day assay using OVCAR-3 (sensitive cell line) and A549 (resistant cell line) to determine cytotoxicity as reported previously.[26]

Hemagglutination and Hemolysis Assays. Human type-O erythrocytes (1.0% in PBS) were treated with various concentrations of TPI and *Tethya* hemolysin and allowed to incubate for 1 h at room temperature. After incubation, the solutions were centrifuged at 1500 rpm for 15 min, the supernatant was removed and mixed with an equal volume of PBS in a cuvette, and hemolysis was quantified by measuring absorbance at 540 nm. Cells lysed hypotonically were used as a positive control. Hemagglutination was determined by allowing a 2% solution of erythrocytes to incubate with various concentrations of TPI and *Tethya* hemolysin; agglutination was determined microscopically at 1, 4,

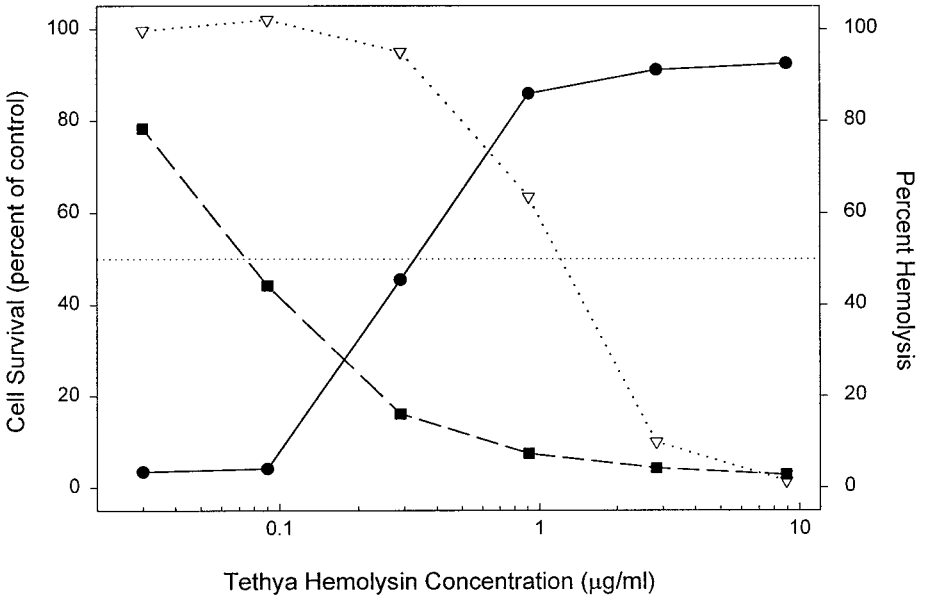

Figure 4. Comparison of the cytotoxic and hemolytic activity of *Tethya* hemolysin. Either human red blood cells (●), A549 human small cell lung carcinoma cells (▽), or OVCAR-3 human ovarian cancer cells (■) were incubated with various concentrations of *Tethya* hemolysin. After a 1-h incubation, cell viability and hemolysis were determined as reported in the Experimental Section.

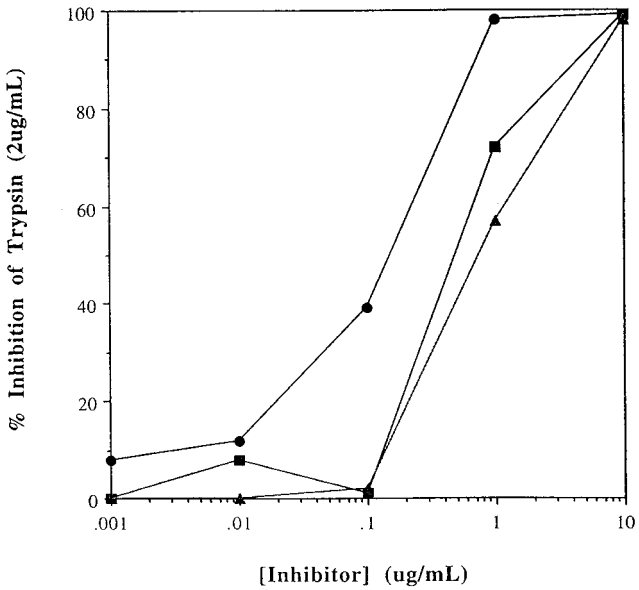

Figure 5. Protease inhibition activity of TPI. Bovine trypsin (2 μg/mL) and 1 mM BAPNA were incubated with log dilutions of either aprotinin (●), soybean tripsin inhibitor (■), or TPI (▲) in 100 mM Tris buffer (pH 8.3) for 35 min at 37 °C. Trypsin activity was measured as the release of *p*-nitroaniline (absorbance at 410 nm) and the percent inhibition was calculated according to the protocol in the Experimental Section.

and 24 h after administration. The agglutinin from *Lens culinaris* (Sigma) was used as a positive control for hemagglutination.

Stability Tests. Crude protein samples from *T. ingalli* were taken up in 50 mM sodium phosphate buffer (pH 7.5) augmented with 0.02% NaN_3 and one of the following components: 10 mM EDTA, 5 mM DTT, and either 1% or 10% glycerol. The final concentration of the protein in solution was 500 μg/mL. Samples treated with glycerol were separated into three aliquots and stored at either −20 °C, 4 °C, or 23 °C for a period of 14 days prior to assay.

To determine pH stability, protein samples, purified through ammonium sulfate precipitation, were taken up in 50 mM of sodium phosphate buffer titrated to the following pH values: 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, and 9.0, and were stored at 4 °C for 24 h prior to assay.

Purification of Sponge Proteins. Lyophilized *T. ingalli* aqueous extract was brought up in DDH_2O at a concentration of 50 mg/mL^{-1} and kept cold on ice. Crystalline ammonium sulfate (Sigma, molecular biology grade) was added to the solution to bring the final mixture to 30% saturation. The mixture was allowed to precipitate on ice for 120 min and was then centrifuged at 3000 rpm for 60 min. The pellets were collected, and the supernatant was then brought to 55% saturation with ammonium sulfate, followed again by precipitation and centrifugation. Finally, the second pellets were collected, and the supernatant was brought to 75% saturation with ammonium sulfate; all other steps were repeated as above.

After ammonium sulfate precipitation, the 30−55% precipitate was brought up in a minimum of 25 mM of sodium phosphate buffer augmented with 0.4 M sodium chloride and 0.02% sodium azide (pH 7.5). The solution was then placed on a Sephadex G-100 column (2.5 × 50 cm) and eluted with the same buffer at a flow rate of 2 mL/min. Fractions were collected every 2 min, and absorbance was measured at 280 nm.

Cytotoxic fractions from the G-100 column, containing *Tethya* hemolysin, were combined and injected (300 μL) onto a G-3000PW gel permeation column (21.5 × 300 mm, TosoHaas) using a BioCad workstation (PerSeptive Biosystems) and eluted with 25 mM of sodium phosphate buffer augmented with 0.2 M sodium chloride and 0.02% sodium azide (pH 7.5). The column was eluted at a flow rate of 5 mL/min, with fractions taken every 30 sec; absorbance was measured at 210 nm. Native molecular weight was determined by calibrating standard proteins (bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (17 kDa), or aprotinin (6.5 kDa) by their retention time (abs 280 nm) and comparing the resulting calibration curve to the retention time of the active protein.

After gel filtration, active fractions were concentrated by sequential ultrafiltration using a 10 kDa molecular weight limit membrane (Amicon), which retained the hemolysin but not TPI, followed by filtration of the 10-kDa filtrate on a 3-kDa membrane which retained TPI.

In an attempt to further purify *Tethya* hemolysin, cytotoxic fractions from the G-3000 column were combined and brought up to 30% saturation with ammonium sulfate prior to application to a Poros phenyl ether hydrophobic interaction column (4.6 × 100 mm, PerSeptive Biosystems) pre-equilibrated with 1.5 M ammonium sulfate and 25 mM sodium phosphate (pH 7.5). The column was washed with 10 column volumes of the starting buffer, and the bound proteins were eluted using a linear gradient from 1.5 M $(NH_4)_2SO_4$ to 0 M $(NH_4)_2SO_4$ over 60 column volumes at a flow rate of 5 mL/min. The column eluate was monitored for conductivity and for absorbance at 210 nm; fractions were collected every 2.5 mL.

Fractions containing TPI, from the G-100 column were combined and applied to a Dynamax C-18 reversed-phase column (3.9 × 300 mm, 5 μ, Rainin). Proteins were bound in a solution of 0.1% aqueous trifluoroacetic acid (TFA) and eluted with the following gradient: 0−20% acetonitrile (0.1% TFA) (4 column volumes), 20−40% acetonitrile (gradient, 10 column volumes), 40−100% acetonitrile (gradient, 6 column volumes) at a flow rate of 10 mL/min. Absorbance was monitored at both 210 and 280 nm.

Red Blood Cell Membrane Binding Studies. Partially purified fractions of *Tethya* hemolysin were incubated with a 1% solution of human red blood cell membranes obtained by hypotonic lysis just prior to use. Extracts were tested at log dilutions and were allowed to incubate for 60 min. After incubation, the mixtures were centrifuged at 15 000 rpm for 20 min, and the supernatants were decanted and tested for cytotoxicity in the two-cell assay system and also analyzed by SDS−PAGE.

Trypsin Inhibition Assay. The assay system used to monitor inhibition of trypsin activity was similar to that previously reported.[27] Briefly, the assay mixture consisted of a 100-mM Tris−HCl buffer (pH 8.3) augmented with 20 mM $CaCl_2$, 1 mM *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA), 2 μg of bovine trypsin, and 20 μL of the inhibitor solution in a final volume of 1.0 mL. Assays were initiated by the addition of the enzyme, incubated at 37 °C for 35 min, and quenched by the addition of 500 μL of 30% HOAc. The resulting solutions were placed in cuvettes and measured for the release of *p*-nitroaniline by absorbance at 410 nm. The percent inhibition was calculated by

$$\% \text{ Inh} = [1 - A_i/A_o] \times 100$$

where A_i and A_o represent the absorbance in the presence and absence of inhibitor, respectively.

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