Quantitative Analysis of Snake Venoms Using Soluble Polymer-based Isotope Labeling*□

Jacob A. Galan‡, Minjie Guo‡, Elda E. Sanchez§, Esteban Cantu§, Alexis Rodriguez-Acosta¶, John C. Perez§, and W. Andy Tao‡¶

We present the design and synthesis of a new quantitative strategy termed soluble polymer-based isotope labeling (SoPIL) and its application as a novel and inclusive method for the identification and relative quantification of individual proteins in complex snake venoms. The SoPIL reagent selectively captures and isolates cysteine-containing peptides, and the subsequent tagged peptides are released and analyzed using nanoflow liquid chromatography-tandem mass spectrometry. The SoPIL strategy was used to quantify venom proteins from two pairs of venomous snakes: Crotalus scutulatus scutulatus type A, C. scutulatus scutulatus type B, Crotalus oreganus helleri, and Bothrops colombiensis. The hemorrhagic, hemolytic, clotting ability, and fibrinogenolytic activities of crude venoms were measured and correlated with differences in protein abundance determined by the SoPIL analysis. The SoPIL approach could provide an efficient and widely applicable tool for quantitative proteomics. *Molecular & Cellular Proteomics 7:785–799, 2008.

The identification and accurate quantification of proteins in high throughput analysis are essential components of proteomics strategies for biomarker discovery and studying cellular functions and processes (1, 2). Although the technology to measure mRNA expression is more established, the measurement of differential protein expression provides a more direct, accurate, and versatile way to detect global changes in cellular dynamics in health and disease (3). Over the past several years, many quantitative techniques and strategies have been introduced and thoroughly examined. The traditional and frequently used method to investigate differential protein abundances on a large scale between samples from different sources is the staining of proteins separated by two-dimensional (2D) PAGE. This method falls short in its reproducibility and its inability to detect low abundance and hydrophobic proteins (4). Recently label-free approaches primarily based on the use of LC and highly accurate mass spectrometers have been investigated (5, 6). These methods, however, rely heavily on computational software for data treatment.

Stable isotopic labeling has remained the most popular method for quantitative proteomics. The introduction of stable isotopes has typically been carried out by (i) chemical derivatization of proteins or peptides, e.g. via the ICAT (7), isobaric tagging for relative and absolute quantitation (Traq) (8), and isotope-coded protein labeling (9) methods; (ii) enzyme catalyzed labeling, e.g. proteolysis in heavy water incorporating $^{18}$O into the newly generated carboxyl-terminal carboxyl groups (10); and (iii) metabolic labeling methods to incorporate isotopically labeled amino acid residues into proteins (stable isotope labeling using amino acids in cell culture (Si-LAC)) (11). ICAT is probably the best described chemical approach. Over the years, both advantages and disadvantages of ICAT have been recognized. Several variations and modifications of ICAT have been attempted to make it more practical and simpler, such as acid-cleavable ICAT reagents (12) and a solid phase based version (13). The adaptation of the solid phase capture and release process is a significant improvement, and the method obviates extra purification steps and has the potential for automation and high throughput experiments. A side-by-side comparison with the ICAT method demonstrated that the solid phase method for stable isotope tagging of peptides is comparatively simpler, more efficient, and more sensitive. However, the most notable disadvantages of the solid phase extraction are the heterogeneous reaction conditions that can exhibit nonlinear kinetic behavior, unequal distribution and/or access to the chemical reaction, and solvation problems. The heterogeneous nature of solid phase reaction for proteomics research presents a serious issue for sample recovery and identification, which usually deal with a small amount of proteins and peptides in extremely complex mixtures.

We attempt to address this issue by the introduction of a

From the ‡Departments of Biochemistry, Chemistry, Medicinal Chemistry, and Molecular Pharmacology, Purdue University, West Lafayette, Indiana 47907, §Natural Toxins Research Center, College of Arts and Science, Texas A&M University-Kingsville, Kingsville, Texas 78363, and ¶Immunochemistry Section, Tropical Medicine Institute, Universidad Central de Venezuela, Caracas 1041, Venezuela Received, July 13, 2007, and in revised form, December 17, 2007 Published, MCP Papers in Press, December 18, 2007 DOI 10.1074/mcp.M700321-MCP200

1 The abbreviations used are: 2D, two-dimensional; SoPIL, soluble polymer-based isotope labeling; PAMAM, polyamidoamine; PLAs, phospholipase A$_2$; MHD, minimal hemorrhagic dose (minimal protein amount that will cause a 10-mm hemorrhagic spot); DMF, $N,N$-dimethylformamide; TCEP, tris(carboxyethyl)phosphine; ACTH, adrenocorticotropic hormone; SCX, strong cation exchange; TPP, Trans-Proteomic Pipeline; MT, Mojave toxin; HT, hemorrhagic toxin; CAM toxin, myotoxin isolated from Crotalus adamanteus venom.
new quantitative strategy and reagents, termed soluble polymer-based isotope labeling (SoPIL). The new reagents are based on soluble nanopolymers such as dendrimers. Dendrimers are a class of hyperbranched synthetic polymers. Built from a series of branches around an inner core, they provide products of different generations and offer intriguing applications (14). Dendrimers are authentic nanoparticles with the dimensions in the range of 2–10 nm. Dendrimers have extremely well defined cascade motifs with a number of characteristics that make them useful in combinatorial chemistry and biological systems. A number of dendrimer types have been used as drug candidates for receptor-ligand interactions, drug carriers for conferring biosurival, and membrane permeability and targeting and have found wide use as carriers for vaccine antigens (14). We introduced dendrimers to proteomics research for the first time in protein phosphorylation analysis (15), but the application of dendrimers in quantitative proteomics, and potentially in vivo proteomics, is still in its early development (16). New applications for dendrimers open an exciting area for proteomics research that will provide a simple and effective alternative to currently available quantitative technologies.

Snake venoms contain complex mixtures of secreted proteins belonging to many different classes such as neurotoxins, κ toxins, cardiotoxins, myotoxins, hemorrhagins, and disintegrins (17). These same proteins that cause tissue damage and trauma when animals or humans are envenomed are also of medical and pharmacological importance. A number of drugs have been derived from snake venom. For example, ancrad from venom of the Malayan pit viper is a fibrinogenolytic enzyme that is used to treat patients after myocardial infarction (18); Aggrastat (tirofiban), first isolated from the venom of the saw-scaled viper, has antiplatelet and anticancer properties (18). Many North American and South American rattlesnakes venoms possess an abundant source of these medically relevant proteins, and new proteins and their activities are being characterized steadily.

One of the most abundant classes of proteins in snake venom is the hemorrhagic toxins. The snake venom hemorrhagic toxins are metalloproteinases that are characterized according to structural domains, such as subclasses P-I, P-II, P-III, and P-IV, that bear structural similarity to either metalloproteinase alone or have domains similar to disintegrins or c-type lectins (19–21). These are zinc-containing metalloproteinases characterized by the presence of a protease domain with additional disintegrins or c-type lectin domains in some of them. They act by degrading the component proteins of the basement membrane underlying capillary endothelial cells. The toxins also act on these cells causing lysis, resulting in hemorrhage. Some of these toxins have been found to exert additional effects such as fibrinogenolysis and platelet aggregation that facilitate hemorrhage (19).

Another highly abundant class of proteins in snake venoms are the phospholipases A₂ (PLA₂s), which are a major component in Viperidae/Crotalidae venom. PLA₂ enzymes are single chain polypeptides of around 120 residues or mixtures of two to five complementary polypeptides (22). These isoenzymes catalyze the hydrolysis of phospholipids into free fatty acids and lysolipids. PLA₂s display a wide variety of neurotoxic, cardiotoxic, myotoxic, hemolytic, convulsive, anticoagulant, antiplatelet, edema-inducing, and tissue-damaging effects (23).

The disintegrins are another important class of molecules that have an active and conserved (RGD or “RGD-like”) sequence structural homology that is located around a hairpin loop (24). The RGD-containing disintegrins show differential binding toward the cell surface receptors known as integrins. Disintegrins can have other active triad motifs (e.g. KGD, MLD, and WGD) that bind and inhibit integrins to varying degrees and can play a role in cell-to-matrix or cell-to-cell inhibition (25). The inhibition of integrins can result in altered signal transduction pathways that influence cell migration or adipogenesis, angiogenesis, and other biological events (26–28).

Disintegrins and other snake venom proteins are found in many species of venomous snakes; however, their abundance in venoms varies from snake to snake. Intrasppecies and interspecies variations in snake venom make a unique biological source of many different interesting toxins, but these variations are also the cause for ineffective antivenoms. One of the most striking examples of interspecies variation in rattlesnake venom occurs in the Mohave rattlesnake, Crotalus scutulatus scutulatus (29–31).

The analysis of venom by proteomics approaches, such as HPLC or 2D gel electrophoresis combined with mass spectrometry, has increased the identification of proteins in the snake venom proteome (9, 32–35). Calvete and co-workers (33) analyzed the venoms of the three subspecies of Sistrurus catenatus (S. catenatus catenatus, S. catenatus tergeminus, and S. catenatus edwardsii) and Sistrurus miliarius barbouri by off-line reverse phase HPLC, amino-terminal sequencing, MALDI-TOF, and CID-MS/MS. They observed a difference in the venom composition of closely related species that have different diets. Birrell et al. (34) studied the diversity of venom proteins from 18 of these snake species, and the venom protein components were separated by 2D PAGE and identified using mass spectrometry and de novo peptide sequenc- ing. These two methods have demonstrated the potential for comparative venom protein analysis. To date, little is known about the relative abundance of snake venom proteins existing in different species or in the same species with different geo-origins. Utilizing stable isotope labeling would provide a more detailed and comprehensible analysis for snake venom variation.

The aim of this study was to describe and characterize the novel quantitative proteomics strategy SoPIL and its applications to examine the venoms from the same species of C. scutulatus scutulatus (types A and B) and to examine the venoms from two geographically unrelated snakes Crotalus
oceanus helleri and Bothrops colombiensis (from North and South America, respectively). Cysteine-containing peptides were efficiently isolated, isotypically labeled, and analyzed by two-dimensional microcapillary LC-tandem mass spectrometry for identification and relative quantification.

**EXPERIMENTAL PROCEDURES**

**Synthesis of the SoPIL Reagents**

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich.

Synthesis of (3,5-Dimethoxy-4-phenylaminomethylphenoxy)butyric Acid (2)—(4-(4-Formyl-3,5-dimethoxyphenoxo)butyric acid (1) (Nova-biochem) (268 mg, 1.0 mmol) was dissolved in 10 ml DMF and MeOH (1:4, v/v) and mixed with aniline (250 mg, 2.5 mmol) and HOAc (75 µl, 1.25 mmol) followed by the addition of NaBH₄CN (120 mg, 2.0 mmol). The reaction proceeded at room temperature for 2 h, 2 ml of H₂O was added followed by evaporation of vacuum, and then the product was extracted with EtOAc and purified by flash chromatography to give NMR pure product 4-(3,5-dimethoxy-4-phenylaminomethylphenoxy)butyric acid (2) (260 mg, 75%). ^1H NMR (CDCl₃, 300 MHz): δ 7.17 (t, J = 7.5 Hz, 2 H), 6.75 (d, J = 7.5 Hz, 2 H), 6.80 (t, J = 7.5 Hz, 1 H), 6.10 (s, 2 H), 4.27 (s, 2 H), 4.00 (t, J = 6.0 Hz, 2 H), 3.80 (s, 6 H), 2.58 (t, J = 7.2 Hz, 2 H), 2.14–2.04 (m, 2 H).

Synthesis of 4-(4-(2-Bromoacetyl(phenylamino)methyl)-3,5-dimethoxyphenoxy)butyric acid (3)—Bromacetyl chloride (Alfa Aesar) (50 µl, 0.6 mmol) in 0.5 ml of anhydrous tetrahydrofuran and 0.6 ml of 1 N NaOH were added dropwise to the tetrahydrofuran solution of compound 2 (174 mg, 0.5 mmol) at 0 °C. The reaction was continued at this temperature for 30 min. Then the reaction mixture was neutralized with 1 N HCl at 0 °C. The product was extracted with EtOAc and purified by flash chromatography to obtain NMR pure product 4-(4-(2-bromoacetyl(phenylamino)methyl)-3,5-dimethoxyphenoxy)butyric acid (3) (175 mg, 75%). ^1H NMR (CDCl₃, 300 MHz): δ 7.26–7.21 (m, 3 H), 6.98–6.97 (m, 2 H), 5.92 (s, 2 H), 4.97 (s, 2 H), 3.96 (t, J = 6 Hz, 2 H), 3.61 (s, 2 H), 3.59 (s, 6 H), 2.58 (t, J = 7.5 Hz, 2 H), 2.11–2.05 (m, 2 H). The heavy isotope form was synthesized in the same way by using [13C]alanine.

**Synthesis of the SoPIL Reagents—PAMAM dendrimer Generation 4.0 (28 mg, 2 µmol) was dissolved in 10 ml of 200 mM MES (pH = 5.8) followed by the addition of 4-pentyenoic acid (1.6 mg, 16 µmol, 6 eq/dendrimer) in 200 µl of DMF, N-hydroxysuccinimide anhydride (20 mg, 15 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl (200 mg, 200 µmol). The solution was stirred at room temperature for 12 h. After intensive dialysis in water, the solution was concentrated by ultrafiltration to 1 ml and split into halves for further reaction with compound 3 and the heavy form, ^12C₀-labeled compound 3, respectively. Terminal alkyn-functionalized G4-PAMAM (1 µmol) prepared above was dissolved in 2 ml of 200 mM MES (pH = 5.8). Then compound 3, either ^12C₀- or ^13C₀-labeled (9.2 mg, 20 µmol), in 2 ml of DMF was added to the above solution followed by the addition of N-hydroxysuccinimide anhydride (10 mg, 15 mmol) and 1-[3-(dimethylaminopropyl)-3-ethylcarbodiimide HCl (100 mg, 100 mM). The reaction was continued at room temperature for 12 h in the dark. After intensive dialysis in water, the solution was concentrated by ultrafiltration to 600 µl, which was directly used for labeling experiments.

**Synthesis of Azide Beads**

1-Amino-11-azido-3,6,9-trioxaundecane (4) (109 mg, 500 µmol) in 500 µl of DMF was added to the beads followed by the addition of 1-hydroxybenzotriazole (63 mg, 500 µmol) and disopropylcarbodiimide (80 µl, 500 µmol). The reaction was allowed to proceed at room temperature overnight, and the beads were extensively washed and dried in a vacuum.

**Synthesis of Cys-specific Solid Phase Beads**

Aminopropyl controlled pore glass beads (100 mg, NH₂, 400 µmol/g) were prewashed with anhydrous DMF. 1-Hydroxybenzotriazole (25 mg, 200 µmol) in 100 µl of DMF, compound 3 (92 mg, 200 µmol) in 600 µl of DMF, and disopropylcarbodiimide (64 µl, 400 µmol) were added to the beads successively for overnight reaction. The beads were then washed sequentially with DMF and dichloromethane, then dried under reduced pressure, and stored at room temperature in the dark.

**Yield Determination of Capturing Cysteine-containing Peptides and Acid Cleavage Reactions Using the SoPIL Reagent and the Direct Solid Phase Approach**

A peptide mixture consisting of 100 pmol of cysteine-containing laminin B (sequence, CDPGYGIGSR) and 20 pmol of non-cysteine containing angiotensin (sequence, DRYYIHPF) was used. Peptides were reduced with 5 mM tris(carboxyethyl)phosphine (TCEP) in 100 µl of 0.1 mM Tris (pH 8.0) and 5 mM EDTA for 10 min at room temperature. Next 10 nmol of ^13C₀ SoPIL reagent or 7 mg of Cys-specific solid phase ^12C₀ beads were used in parallel to capture peptides in a total volume of 100 µl under constant agitation. Aliquots of 1 µl of supernatant were drawn from the reaction mixture for MS analysis before the start of reactions and at different time points during the reaction. After 1 h of incubation, the reactions were quenched by the addition of 2 µl of 200 mM DTT for 5 min. For the reaction with the SoPIL reagent, 10 mg of azide beads was added in a total volume of 400 µl of solution containing 2.5 mM TCEP, 2.5 mM CuSO₄, 0.25 mM tris(triazolyl)amine for 30 min. Both beads were combined and washed successively by 1 mM NaCl, H₂O, and 80% acetonitrile twice. One hundred microliters of 90% TFA in water was added to the beads and incubated for 1 h. The released peptides were collected by filtration, and beads were washed with 80% acetonitrile twice. The elutions were combined and dried under vacuum for MS analysis. Mass spectra were acquired at the MS mode using a MALDI-TOF/TOF mass spectrometer (ABI 4700, Applied Biosystems, Foster City, CA). Measurements were performed with a 200-Hz solid state laser in positive reflector mode with a 2.5-kV acceleration voltage. For each MS spectrum, 1000 laser shots in an m/z window of 800–3000 were accumulated using Data Explorer version 4.2 (Applied Biosystems) software. Mass calibration was achieved using the Sigma calibration kit that includes reference peptides angiotensin I, ACTH, bradykinin, and fibrinopeptide. For sample preparation 0.3 µl of aliquots of the solution was spotted on a target and mixed 1:1 with a matrix consisting of 80% acetonitrile in water, 0.1% TFA, and 7 mg/ml α-cyano-4-hydroxycinnamic acid.

**Venom Collection**

Individual Mohave rattlesnake (C. scutulatus scutulatus) types A and B (Avid 011-032-076 and 011-064-358) were collected in Culberson County, Texas and Pinal County, Arizona, respectively. A Southern Pacific rattlesnake (Avid 011-032-076) was collected from Southern California, San Bernardino County. These snakes are currently housed at the Natural Toxins Research Center, Texas A&M University-Kingsville, Kingsville, TX. The B. colombiensis is currently housed at the Instituto de Medicina Tropical, Universidad Central de Vene-
zuella, Caracas, Venezuela. Venom from each snake was extracted in their corresponding locations by allowing the snakes to bite into Parafilm stretched over a disposable plastic cup. Each venom sample was centrifuged (500 x g for 10 min), filtered through a 0.45-μm filter under positive pressure, and frozen at -90 °C until lyophilized.

### Venom Preparation

Fifty microliters of a 10 mg/ml snake venom solution was prepared, denatured using 8 M urea, and reduced with 5 mM TCEP for 30 min at 37 °C. Urea in the samples was then diluted 4 times with 200 mM Tris, pH 8.5. Twenty micrograms of sequence grade trypsin (Promega) was added and incubated at 37 °C for 16 h. The samples were then dried to 100 μl.

### Isotopic Labeling and Isolation of Cysteine-containing Peptides

Forty microliters of digested peptide mixture from each sample was labeled with 30 nmol of SoPIL reagent. The venom peptides from Mohave type A and Southern Pacific rattlesnakes were labeled using 12C6SoPIL, and venom peptides from Mohave type B and mapanare were labeled with 13C6SoPIL for 2 h. Then 20 μl of 200 mM DTT was added to each mixture to quench the excessive bromoacetyl group on the dendrimer surface. The two mixtures (Mohave types A and B; Southern Pacific and mapanare) were then mixed together followed by the addition of 130 μl of H2O, 50 μl of 20 mM tris(triazolyl)amine, and 20 μl of 50 mM CuSO4, in a final volume of 400 μl and TCEP, CuSO4, and tris(triazolyl)amine concentration of 2.5, 2.5, and 0.25 mM, respectively. The mixtures were then incubated with 5 mg of azide beads at room temperature for 1 h. The beads were washed with 50 mM Tris, 1 M NaCl, H2O, and 80% acetonitrile three times, respectively. The beads were incubated with 100 μl of 90% TFA for 1 h. The released peptides were collected using filtration, and beads were washed with 80% acetonitrile twice. The elutions were combined and dried with a centrifugal SpeedVac.

### Nanoflow LC-MS/MS Analysis

The analysis was performed on an Agilent 1100 nanoflow (Agilent Technologies, Wilmington, DE) connected to an LTQ linear ion trap mass spectrometer (ThermoFisher, San Jose, CA). The dried peptides were reconstituted in 16 μl of 0.1% formic acid and introduced on the nanoflow system with two-dimensional liquid chromatography (SCX and C18 reverse phase). The strong cation exchange chromatography (one-dimensional) was performed using an Agilent SCX (Zorbax, 3.5 μm, 35 × 0.3-mm, Agilent Technologies) column. The buffer used was 0.1% formic acid with the eluting buffer containing 10 different molar concentrations of 20, 40, 60, 80, 100, 150, 200, 300, 500, and 1000 mM ammonium acetate. The C18 reverse phase LC (2D) was performed using an IntegraFrit™ (50-cm × 75-mm) capillary column (New Objectives) packed with 5-μm C18 Magic® beads (Michrom Bioresources, Inc.; 75-μm inner diameter and 12 cm of bed length). The electrospray ionization emitter tip was generated with a laser puller (Model P-2000, Sutter Instrument Co.). The mass spectrometer was operated in the data-dependent mode in which a full-scan MS was followed by MS/MS scans of the 10 most abundant ions with +2 to +3 charge states. The mass exclusion time was 180 s.

### Data Analysis

The MS/MS data were converted to a mzXML format using the open source Trans-Proteomic Pipeline (TPP) software (Version 2.9.4), and the resulting mzXML files were searched against the Swiss Protein Database (Version 43.0 with 146,720 entries) using the SEQUEST™ algorithm on the Sorcerer™ integrated data appliances (IDA) server (Software Version 2.5.6; SageN, Inc., San Jose, CA). Peptide mass tolerance was set at 3.0 amu, and MS/MS tolerance was set internally by the software with the values varying from 0 up to 1 amu. Search criteria included a static modification of cysteine residue of 133 Da (mass of cysteine plus light SoPIL reagent tag) and a variable modification of 6 Da for cysteines (for the heavy SoPIL reagent tag) and methionine oxidation (16 Da). Searches were performed with semitryptic digestion and allowed a maximum of two missed cleavages on the peptides analyzed from the sequence database. The validation of protein identification and quantification were performed with the TPP software. The TPP software includes a peptide probability score program, PeptideProphet (37), that aids in the assignment of peptide MS spectrum and the ProteinProphet program that assigns and groups peptides to a unique protein or a protein family if the peptide is shared among several isoforms (38). Because snake genome has not been sequenced and snake venom has a high sequence homology in the cysteine-containing region, a number of peptides belong to more than a single protein. ProteinProphet (38) partially solved the problem by grouping all identified peptides and adjusting the corresponding probability score. However, based on current knowledge of snake venom there are still quite a few ambiguous cases, and therefore peptides were assigned to multiple protein identifications (supplemental Tables S1–S4). The PeptideProphet program uses various SEQUEST scores (e.g., Xcorr) and a number of other parameters to calculate a probability score for each identified peptide. PeptideProphet allows filtering of large scale data sets with assessment of predictable sensitivity and false positive rates. A PeptideProphet and ProteinProphet threshold of 0.9 probability score was used for all accepted SoPIL reagent-labeled peptide identifications and protein assignment (the estimated false positive rate is 1.1% for a probability score of 0.9). Peptide quantifications were analyzed using ASAPRatio software (39), which reconstructs ion chromatograms from SoPIL reagent-labeled peaks and performs automated statistical analysis of peptide abundance ratios. The ASAPRatio program reconstructs a raw single ion chromatogram by summing all ion intensities within an m/z range covering the first three theoretical isotopic peaks of the peptide and over the chromatographic elution period of the peptide. It then applies the Savitzky-Golay smooth filtering method to obtain a smoothed chromatogram. The peptide elution peak is identified along with the corresponding peak center and peak width from the smoothed chromatogram (39). The quantification of peptides identified in different SCX elutions was averaged with manual inspection. All quantified peptides were manually inspected and verified for authenticity. Information about PeptideProphet, ProteinProphet, and ASAPRatio programs and other programs in Trans-Proteomic Pipeline is available on line.

### Hemorrhagic Activity

The method of Omori-Satoh et al. (40) was used to determine the minimal hemorrhagic dose (MHD) for the crude venoms. One milligram per milliliter solution was prepared for each snake venom. Eight one-half series dilutions were prepared for the snake venoms (1 to 1/512), of which 0.1 ml of each dilution was injected intracutaneously into the depilated backs of rabbits. After 24 h, the rabbit was sacrificed, and the skin was removed. A caliper was used to measure the hemorrhagic spot diameter on the skin, and the MHD was determined. The MHD is defined as the amount of venom protein that causes a 10-mm hemorrhagic spot.

### Sonoclot Analyzer Profiles

A glass bead-activated test (gbACT™ kit obtained from Sienco, Inc.) was used to monitor the effect of venoms on human blood coagulation on a Sonoclot® Coagulation and Platelet Function Analyzer (Sienco, Inc.) according to Sanchez et al. (31). Data acquisition and
analysis were performed with Signature Viewer software (Sienco, Inc.).

Removal of Serine Proteases from Crude Venoms

A HiTrap benzamidine FF (fast flow) column was used to remove the serine proteases from the crude venoms. One-milliliter (5 mg/ml) venom samples in 0.05 M Tris-HCl, 0.5 M NaCl, pH 7.5 were added to a 1-ml HiTrap column using a 1-ml luer lock syringe. The serine proteinase-free venom was washed out using 0.05 M Tris-HCl, 0.5 M NaCl, pH 7.5. The serine proteinases were eluted off with 0.01 M HCl, 0.5 M NaCl, pH 2.0 in 1-ml aliquots in Eppendorf tubes containing 70 μl of 1 M Tris-HCl, pH 9.0.

Fibrinogenolytic Activity

Fibrinogenolytic activity of crude and serine protease-free snake venom (0.03 mg/ml) was determined using 12% Tris-glycine gels on a PowerPac Basic\textsuperscript{TM} (Bio-Rad). Twenty microilters of fibrinogen solution at 2.5 mg/ml in 0.05 M Tris-HCl, pH 7.5, containing 0.5 M NaCl with 10 μl of the venom samples was incubated at 37 °C for 2 and 24 h. A total of 12 μl of the fibrinogen/venom mixture was reduced with 6 μl of Tris-glycine SDS sample buffer (2×) and 2 μl of NuPage sample reducing agent (10×) and boiled for 3 min. The gels were stained with SimplyBlue\textsuperscript{TM} SafeStain (Invitrogen). The proteolytic activity was determined by the disappearance of the α, β, and γ chains of the fibrinogen.

Hemolytic Activity

The minimal hemolytic activity of the crude venoms was determined as described by Habermann and Hardt (41) with modifications. Briefly, 0.3 ml of packed human erythrocytes were washed five times with saline solution, and 0.3 ml of a large, fresh egg yolk diluted 1:4 with saline solution and 0.25 ml of a 0.01 M CaCl\textsubscript{2} solution were added to 25 ml of 0.8% agarose dissolved in PBS solution (pH 8.1). The mixture was poured into plastic Petri dishes (135 × 80 mm) and allowed to solidify. After the mixture was solidified, 12 3-mm-diameter wells were prepared and filled with 10 μl of crude venom solution at various concentrations. The plates were incubated at 37 °C for 5 h, and the diameters of the hemolytic haloes were measured. Saline solution and PBS were used as controls. The minimal hemolytic dose was defined as the amount of venom protein that causes a 10-mm halo spot.

RESULTS AND DISCUSSION

**Rationale for the Use of Soluble Nanopolymer-based Reagents for Quantitative Proteomics**—We attempt to address two concerns in existing proteomics research with the SoPIL reagents. The first one is the efficiency and consistency of sample preparation in proteomics research. Sample preparation in proteomics includes the isolation and labeling of a class of proteins/peptides from complex mixtures. Current methods either include extra purification steps that could lead to severe sample loss or solid phase extraction that is usually limited by heterogeneous reaction and nonlinear kinetics. We address this issue by building the function groups for reaction, isotopic labeling, and isolation on a soluble nanopolymer. The soluble polymer, PAMAM dendrimer G4, was functionalized with the reactive group-bromoacetyl group for site-specific, stable isotopic labeling of cysteine-containing peptides, [\textsuperscript{13}C\textsubscript{2}]aniline or [\textsuperscript{13}C\textsubscript{4}]aniline as the isotope tag, and pentynyl group as the “handle” for the isolation of polymer-bound peptides through the highly efficient click chemistry (Scheme 1). The design was based on the concept that specific capture of targeted proteins/peptides, the rate-limiting step, is more efficient when carried out in the solution phase than on the solid phase, and in the second step, samples tagged with nanopolymers are isolated on a solid phase by choosing a highly efficient bioconjugation reaction between a pair of bioorthogonal groups on the soluble polymer and on the solid phase (Scheme 2). The high ratio of the reactive groups to the handle groups in a homogenous reaction enables us to tag the samples without extra purification steps to remove excessive reagents that are normally required for small chemical reagents such as ICAT reagents.

Soluble nanopolymers such as dendrimers also provide another unique feature for proteomics research. Dendrimers can effectively permeate into living cells and have become one important class of molecules for drug and gene delivery. Although this feature was not explored in this study, SoPIL could address the second concern that in vitro proteomics preparations can only, at best, approximate the functional state of proteins in the living cell or organism by achieving proteomics in living cells or in vivo.

**Characterization and Application of the SoPIL Method to Standard Peptide and Protein Mixtures**—We have investigated several types of biologically inert coupling partners functionalized on the PAMAM dendrimer and on the solid phase separately that would react at practical rates at submillimolar concentrations at high yield. We chose to use click chemistry, Cu(I)-catalyzed azide-alkyne cycloaddition, because of its biocompatibility and high reaction efficiency. The...
syntheses of SoPIL reagents and azide solid phase beads and the chemical procedure to tag Cys-containing peptides are illustrated in Schemes 1, 3, and 4, respectively. To isotopically label and then recover tagged peptides for quantitative mass spectrometry analysis, an acid-cleavable linker, 5-(2-formyl-3,5-dimethoxyphenoxy)pentanoic acid (backbone amide linker), was introduced between the dendrimer and the isotope tag.

To demonstrate the SoPIL strategy and to make a parallel comparison between the SoPIL and one-step solid phase methods, we used a standard peptide mixture consisting of a cysteine-containing laminin B (m/z 967) and a non-cysteine-containing angiotensin II (m/z 1046) (Fig. 1A). To make accurate comparisons, we designed a method based on stable isotope dilution. The standard peptide mixture was allowed to react with the light SoPIL reagent (SoPIL 12C6) and the heavy solid phase reagent (solid phase 13C6) in parallel. The solid phase reagent was synthesized in a similar fashion to directly incorporate the acid-cleavable linker, the isotope tag aniline, and the bromoacetyl group as the thiol-specific group on the aminopropyl controlled pore glass beads (Scheme 3) (see “Experimental Procedures” for detailed synthesis). Laminin B was attached to the polymer in less than 1 min after the SoPIL reagent was added into the peptide mixture. In contrast, only 50% of laminin B was captured on the solid phase during the same time frame, and it took over 30 min for the solid phase reagent to completely capture the peptide in the solution (Fig. 1, B and C). Both reactions were allowed to go to completion and quenched with 10 mM DTT. The SoPIL reagent was then captured on the azide-functionalized beads through the click chemistry. The resulting beads were combined with solid phase 13C6 beads, washed, and treated with acid. Recovered tagged products were analyzed on the same MS spectrum. The yield using the SoPIL method was over 85%, whereas the yield was less than 40% for the solid phase method (Fig. 1D). Therefore, the data demonstrated that the capture and release of laminin B using the SoPIL reagent were specific and more efficient than...
the SoPIL reagents. Using 1–400 fmol, all four proteins were explicitly identified with multiple cysteine-containing peptides that were accurately quantified with mean differences below 20% between the observed and expected quantities. In addition, the SoPIL reagents efficiently labeled peptides containing more than one cysteine residue, a feature the solid phase method cannot achieve due to steric hindrance (13).

Potential modifications on other amino acid residues (e.g., lysine, histidine, methionine, and tryptophan) were examined using differential modifications on these residues during database search (supplemental data). No other modifications were identified except cysteine residues, indicating that the derivatization using the SoPIL reagents is specific.

Application of the SoPIL Method to the Quantitative Analysis of Snake Venoms—The SoPIL strategy was applied to study differences in protein abundance in snake venoms. We chose to quantitatively analyze two pairs of snake venoms: the first pair, Mohave rattlesnake venom types A and B from the same species, and the second pair, B. colombiensis and C. oreganus helleri, two regionally distinctive snakes. Many snake venom proteins are extremely cysteine-rich (18), making them an excellent paradigm for us to study using cysteine-specific SoPIL reagents.

Most venoms from the Mohave rattlesnakes in the southwestern United States and in Mexico, C. scutulatus scutulatus

| Protein name       | Peptide sequence   | Observed ratio$^a$ | Mean ± S.D. | Expected ratio | Error |
|--------------------|--------------------|--------------------|-------------|----------------|-------|
| Bovine serum albumin| K↓ TC*VADESHAGC*EK↓ S | 1.33 ± 0.22        | 1.19 ± 0.22 | 1.0            | 19    |
|                    | F↓ HADIC*LTPDTEK↓ Q | 1.03 ± 0.14        |             |                |       |
|                    | K↓ YIC*DNQDTISSK↓ L | 1.17 ± 0.2         |             |                |       |
|                    | R↓ NEC*FLSHKDDSPDLPK↓ L | 1.44 ± 0.51   |             |                |       |
|                    | K↓ DDPHAC*YSTVFDK↓ L | 1.29 ± 0.24        |             |                |       |
|                    | K↓ EAC*FAVEGPK↓ L   | 1.18 ± 0.16        |             |                |       |
|                    | K↓ LKEC*C*DKPLEEK↓ S | 1.61 ± 0.07        |             |                |       |
|                    | R↓ ETYGDMADC*C*EK↓ Q | 1.32 ± 0.21        |             |                |       |
|                    | K↓ SLHTLFGGDEL*C*K↓ V | 0.88 ± 0.15    |             |                |       |
|                    | K↓ EYEATLLEE*C*AK↓ D | 1.12 ± 0.17        |             |                |       |
|                    | K↓ SLHTLFGGDEL*C*K↓ V | 0.88 ± 0.15        |             |                |       |
|                    | K↓ YNGVFAQC*C*QAEDK↓ G | 1.48 ± 0.22       |             |                |       |
|                    | K↓ LFTFHIADIC*TLTPDTEK↓ Q | 1.15 ± 0.14     |             |                |       |
|                    | K↓ EC*C*HQDLLEGC*ADDRLAK↓ Y | 1.02 ± 0.54 |             |                |       |
|                    | L↓ SFNPTQLEEOC*HI↓ F | 0.62 ± 0.08        | 0.52 ± 0.11 | 0.5            | 4     |
| β-Lactoglobulin    | R↓ LSFNPTQLEEOC*HI↓ F | 0.46 ± 0.08        |             |                |       |
|                    | F↓ C*MENSAEEOSLAC*C*QVLR↓ T | 0.47 ± 0.06 |             |                |       |
| α-Lactalbumin      | K↓ FLDDDDLDDMC*VK↓ K | 1.9 ± 0.78         | 2.15 ± 0.43 | 2.0            | 8     |
|                    | K↓ DDQNHSSNIC*NISC*DK↓ F | 2.74 ± 0.56     |             |                |       |
|                    | H↓ SSNIC*NISC*DK↓ F | 1.82 ± 0.19        |             |                |       |
| Lysozyme C         | L↓ LSSDDTASVNC*AK↓ K | 0.99 ± 0.15        | 1.07 ± 0.12 | 1.0            | 7     |
|                    | C↓ SALLSSDDTASVNC*AK↓ K | 1.16 ± 0.3     |             |                |       |
|                    | R↓ NLC*NIPC*SALLSSDDTASVNC*AK↓ K | 0.94 ± 0.15 |             |                |       |
|                    | R↓ C*ELAAAAMK↓ R | 1.22 ± 0.05        |             |                |       |

$^a$↓ indicates a tryptic cleavage site; dash indicates a carboxyl-terminal residue. * indicates the modification of Cys residue by SoPIL reagents.

$^b$ Ratios ($^{12}\text{C}_\text{iso}^{13}\text{C}_\text{bio}$) and errors were calculated using the open source ASAPRatio program.
Quantitative Snake Venom Proteomics

### Table II
Partial list of proteins identified and relative abundance in the venom of *C. scutulatus scutulatus* A and B

| Entry no. | Protein ID | Description                                                                 | No. of identified peptides | Mean ± S.D. a |
|-----------|------------|------------------------------------------------------------------------------|----------------------------|---------------|
| 1         | gi|13959630|sp|Q91053|VSP1_AGKCA  | Calobin precursor                               | 8  | 1.84 ± 0.76 |
| 2         | gi|461932|sp|P30403|DISR_AGKRH  | Hemorrhagic protein-rhodostomin precursor (RHO) (contains disintegrin rhodostomin (disintegrin kistrin)) | 2  | 0.23 ± 0.08 |
| 3         | gi|26397690|sp|Q8UVZ7|PA2H_CROAT  | Phospholipase A₂ homolog Gax-K49 precursor       | 2  | 0.51 ± 0.45 |
| 4         | gi|13959639|sp|Q9DF67|VSP2_TRUE  | Venom serine proteinase 2 precursor (SP2)         | 6  | 2.89 ± 0.76 |
| 5         | gi|6093836|sp|Q93364|OXLA_CROAD  | l-Amino-acid oxidase precursor (LAO) (LAOO) (apo) oxin I | 18 | 0.58 ± 0.42 |
| 6         | gi|27151648|sp|O42188|PA29_AGKHP  | Phospholipase A₂ homolog                           | 2  | <0.05         |
| 7         | gi|462320|sp|P34182|HRTE_CROAT  | Hemorrhagic metalloproteinase HT-E precursor      | 6  | 0.15 ± 0.46 |
| 8         | gi|231997|sp|P30431|DISJ_BOTJA  | Putative venom metalloproteinase jararhagin precursor (HF2-proteinase) (contains disintegrin)       | 19 | 0.25 ± 0.36 |
| 9         | gi|13959659|sp|Q9YG16|VSP2_AGKHP  | Palladin 2 precursor                               | 5  | 2.49 ± 0.97  |
| 10        | gi|122973|sp|P07973|HEMA_INCEN  | Hemagglutinin-esterase precursor (contains hemagglutinin chain 1 (HE1))                            | 1  | 5.19 ± 0.84  |
| 11        | gi|13959638|sp|Q9DF66|VSP3_TRUE  | Venom serine proteinase 3 precursor (SP3)          | 12 | 0.27 ± 0.52  |
| 12        | gi|129470|sp|P24027|PA2C_CRODU  | Phospholipase A₂ CB2 precursor (crototoxin basic chain 2) (phosphatidylcholine 2-acylhydrolase)      | 6  | 2.66 ± 3.42  |
| 13        | gi|26007015|sp|P18998|PA2A_CROSS  | Mojave toxin acidic chain precursor (Mtx-a)        | 11 | 1.35 ± 1.26  |
| 14        | gi|118621|sp|P21858|DISI_AGKHA  | Disintegrin halysis (platelet aggregation activation inhibitor)                                 | 1  | 0.15 ± 0.02  |
| 15        | gi|462301|sp|P20164|HR1B_TRIFL  | Hemorrhagic metalloproteinase HR1B (trimerelisin I)                                                 | 2  | 0.11 ± 0.13  |
| 16        | gi|27151653|sp|P9QVE9|PA2C_AGKRP  | Phospholipase A₂ S1E6-c precursor (phosphatidylcholine 2-acylhydrolase)                           | 5  | 0.07 ± 0.09  |
| 17        | gi|118652|sp|P16338|DISI_AGKPI  | Disintegrin apilagin (platelet aggregation activation inhibitor)                                     | 1  | <0.05         |
| 18        | gi|13959631|sp|Q91507|VSP1_TRIMU  | Murocibrase 1 precursor, murocifrase 3 precursor                                                      | 3  | 1.20 ± 0.22  |
| 19        | gi|118681|sp|P21859|DISI_TRIFL  | Disintegrin triflavin (platelet aggregation activation inhibitor)                                      | 7  | 0.46 ± 0.24  |
| 20        | gi|129398|sp|P04417|PA21_AGKHA  | Phospholipase A₂, basic (PA2-I) (Phosphatidylcholine 2-acylhydrolase)                              | 2  | <0.05         |
| 21        | gi|127777|sp|P12028|MYX1_CROVC  | Myotoxin I, myotoxin (toxic peptide C), myotoxins 2 and 3, myotoxin (CAM toxin)                     | 1  | 1.39 ± 0.23  |
| 22        | gi|125179|sp|P15946|KLKB_MOUSE  | Glandular kalliakrein K11 precursor (tissue kalliakrein) (mGK-11)                                  | 1  | 2.08 ± 2.24  |
| 23        | gi|27734437|sp|P59171|PA25_ECHOC  | Phospholipase A₂ 5 precursor (phosphatidylcholine 2-acylhydrolase)                                | 1  | 0.55 ± 0.03  |
| 24        | gi|13959617|sp|O13059|VSP1_TRIGA  | Venom serine proteinase 1 precursor                    | 3  | 1.41 ± 0.20  |
| 25        | gi|13959616|sp|O13058|VSP3_TRIFL  | Venom serine proteinase 3 precursor                    | 3  | 1.79 ± 0.65  |
| 26        | gi|3122187|sp|P81176|VSP1_AGKCA  | Halystase                                           | 4  | 0.04 ± 0.00  |

a Large standard deviation is mainly due to the effect of endogenous proteolytic activities and degenerate peptides.

dominated by a number of highly abundant proteins. Our analysis also revealed peptide redundancy that could be due to the high sequence homology conserved in the cysteine-containing region unique to snake venom. The distribution of identified proteins is illustrated in Fig. 2A. Approximately 18% of identified proteins were classified as homologs. Consistent with previous reports on the Mohave rattlesnake, the quantitative measurements indicated that several classes of cysteine-rich proteins dominantly exist in venom A but not in venom B, such as Mojave toxins. The Mohave rattlesnake is an example of extreme intraspecies variation in venom characterization (42). Mohave rattlesnake venom is one of the most toxic snake venoms found in the United States. The lethal dose killing 50% of a mouse population (LD₅₀) ranges...
between 0.13 and 0.54 mg/kg of body mass (43). This potent lethality is largely due to Mojave toxin, a powerful presynaptically acting neurotoxin that blocks the neurotransmitter acetylcholine resulting in paralysis. Quantitative proteomics analysis determined that the relative intensity of Mojave toxin α subunit in the C. scutulatus scutulatus type A venom is 15 times more abundant than that in the type B venom (Fig. 3, A and B).

Conversely the disintegrins were only observed in venom B, which was verified in a recent study by Sanchez et al. (31). The disintegrin protein in Mohave type B venom was characterized and showed a strong correlation with the crude venom from these snakes. Hemorrhagic activity for Mohave type B is higher than type A (31). In contrast, Mohave type A was not hemorrhagic and did not have the genes for disintegrins, which were probed with primers design for a highly conserved Crotalus atrox disintegrin gene and analyzed using PCR. This study also demonstrated that the disintegrin protein was also inclusive to Mohave type B venom and not type A. The proteomics data showed that type B had more hemorrhagins (venom metalloproteinases), thrombin-like enzymes (serine proteinases), and platelet-aggregating proteinases, which promote and facilitate degradation, hemolysis of cell structural membranes, and inhibition of platelets resulting in tissue damage leading to excessive hemorrhaging. These results were also confirmed by the fibrinogenolytic gels in which both metallo- and serine proteinases were responsible for cleaving the α and the β chain, respectively (Fig. 4, A and B). Many thrombin-like enzymes were also found in the Mohave type A venom, such as serine proteinases, venombin A, and calobin. Our results also showed that a snake venom metalloproteinases with a disintegrin domain exist (domain conserved in acostatin-B, piscivostatin-β, and contortrostatin) in the Mohave type A venom. However, the serine proteinases were the ones responsible for the cleavage of the β chain of fibrinogen (Fig. 4, A and B).

The Southern Pacific rattlesnake (C. oreganus helleri) and Mapanare (B. colombiensis), which are indigenous to North and South America, respectively, display different venom characterizations. Most Bothrops species in South America cause necrosis, hemorrhage, and procoagulant activity induc-
Fig. 4. Fibrinogenolytic activity of venoms at 2 and 24 h. Ten microliters of whole (A and C) and serine proteinase-
free (B and D) venom (0.03 mg/ml) was added to 20 μl of fibrinogen solution (2.5 mg/ml) in 0.05 M Tris-HCl at pH 8.5 and incubated at 37 °C for 2 and 24 h. Twenty-four microliters of fibrinogen/venom fraction mixture was added to 12 μl of SDS buffer + 4 μl of reducing agent from Invitrogen and boiled for 3 min. Twenty microliters of the mixture was added into the wells of 12% Tris-glycine gels. Lane 1, fibrinogen control; lane 2, C. scutula-
tus scutulatus type A; lane 3, C. scutula-
tus scutulatus type B; lane 4, C. orega-
num helleri; and lane 5, B. colombiensis. Gels were run at 125 V, 40 mA for 1.5 h using a Bio-Rad PowerPac Basic. Gels were stained with SimplyBlue SafeStain.

of envenomation (45, 46). The same amounts of snake ven-
om of B. colombiensis and C. oreganus helleri were also labeled by light and heavy SoPIL reagents, respectively; combined; and processed as described in Scheme 4. This second set for SoPIL analysis identified and quantified a similar num-
ber of peptides and proteins in the Swiss Protein Database (Table III and supplemental Tables S3 and S4). The distribu-
tion of identified proteins is illustrated in Fig 2B. Similar to the first Mohave rattlesnake analysis, 22% of identified proteins were classified as homologs.

Myotoxins are typically small proteins and peptides in the snake venom that upon envenomation can induce irreversible damage to skeletal muscle fibers (myonecrosis) (47). They are abundant and widespread in South American venomous snakes but can also be found in the venoms from other species, including the timber rattlesnake (Crotalus horridus) found in North America (48). In the analysis, myotoxins I, II, and III were more abundant in B. colombiensis venom than in the C. oreganus helleri venom. In contrast, CAM toxin, a myotoxin, was found in C. oreganus helleri venom. In addition, phospholipase A₂ α (phosphatidylcholine 2-acylhydrolase) was also present in the venom of C. oreganus helleri venom. These results are consistent with the previous study in which South American Bothrops species exhibit increased procoagulant and myotoxic activity compared with many North American Crotalus species (49). Neurotoxic PLA₂ myotoxins can also be present in a number of viperid/crotalid species, such as cro-
toxin, a neurotoxin found in the venom of Crotalus durissus terrificus from South America (50).

The Southern Pacific rattlesnake, C. oreganus helleri, has myotoxic, neurotoxic, and hemorrhagic components in its venom (51, 52). French et al. (53) reported that Mojave toxin (MT) has been detected in five of 25 C. oreganus helleri using anti-MT antibodies and was confirmed using nucleotide se-
quence analysis. All of the positive venom samples for MT were collected from Mt. San Jacinto in Riverside County, California. In our study, no MT was found in the venom of the Southern Pacific venom. Hemorrhagic metalloproteinase HT-E precursors (atrolysins E, D, and C) were the most abun-
dant proteins relative to the mapanare venom in addition to adamanysin II (proteinase II). The minimal hemorrhagic dose (minimal protein amount that will cause a 10-mm hemorrhagic spot) for the Southern Pacific venom has been reported to be 2.25 μg (54).

Crude Snake Venom Biological Assays—The biological as-
says, hemorrhagic, hemolytic, Sonoclot, and fibrinogenolytic, were carried out on the four crude snake venoms, and these assay results were compared with those obtained with the SoPIL method. In the venom of C. scutulatus scutulatus type A, the MHD was 162.5 μg, and conversely type B venom had an MHD of 16 μg. This assay confirms the higher abundance of metalloproteases existing in type B venom relative to type A analyzed using the SoPIL method (Table II). In addition, the hemolytic activity of type B venom was higher than type A, possibly due to the higher abundance of most phospho-
lipases found in type B venom, as confirmed by the SoPIL method. Furthermore the Sonoclot analyzer revealed that Mo-
have type B venom had a delayed activated clot time of 354 s...
with a low clot rate of 6.2 clot signals/min, whereas Mohave type A venom displayed a normal activated clot time of 144 s with a low clot rate of 10 clot signals/min (Fig 5A and Table IV). These results are in accordance with the SoPIL method in which more metalloproteins were found in type B venom than in type A. Furthermore the α chain of fibrinogen was cleaved by metalloproteases found in type B venom but not in type A venom (Fig. 4, A and B). Metalloproteases inhibit blood coagulation (23), and they can be α- or β-fibrinogenases depending on their specificity to cleave the α or β chain of fibrinogen (55). Serine proteases are other types of proteins found in snake venom that affect platelet aggregation, blood coagulation, fibrinolysis, the complement system, blood pressure, and the nervous system (23, 56–62). Serine proteases were found in slightly higher abundance in Mohave type A venom according to the SoPIL method (Table II). According to the results in the fibrinogenolytic assay, both type A and B venoms contained serine proteases that cleaved the β chains (Fig. 4, A and B); this was evident when the serine proteases were removed from these venoms and no cleavage of β chain was observed (Fig. 4B). However, there is further evidence that both metallo- and serine proteases in both type A and type B venoms are acting complementarily on the β chains. For instance, whole venom in 2 h was able to cleave β chain, but when serine proteases were removed the β chains remained, suggesting that serine pro-

### Table III

Partial list of proteins identified and relative abundance in the venom of *C. oreganus helleri* and *B. colombiensis*

| Entry no. | Protein ID | Description | No. of identified peptides | Mean ± S.D. |
|-----------|------------|-------------|---------------------------|-------------|
| 1         | gi|34922459|P83519|LEC2_BOTJR galactose-specific lectin (BJcul) | 2 | 0.14 ± 0.04 |
| 2         | gi|6093636|P093364|OXLA_CROAD L-Amino-ácid oxidase precursor (LAO) (LAO) (axopin I) | 17 | 3.61 ± 2.42 |
| 3         | gi|1171971|PA21_BOTJR Phospholipase A, precursor (phosphatidylcholine 2-acylhydrolase) (BJUPLA2) | 7 | 0.13 ± 0.21 |
| 4         | gi|27151652|O24129|PA28_AGKHP Phospholipase A, A* (phosphatidylcholine 2-acylhydrolase) | 4 | 0.40 ± 0.58 |
| 5         | gi|13959633|Q91509|VSP3_TRIMU Mucrofibrase 3 precursor | 2 | 1.45 ± 0.75 |
| 6         | gi|584725|P34179|ADAM_CROAD Adamalysin II (proteinase II) | 8 | 2.18 ± 2.00 |
| 7         | gi|231997|P30431|DISJ_BOTJA Putative venom metalloproteinase jararhagin precursor (HF2 proteinase) (contains disintegrin) | 21 | 2.53 ± 4.56 |
| 8         | gi|13959659|Q9YG16|VSP2_AGKHP Palladin 2 precursor | 4 | 0.24 ± 0.87 |
| 9         | gi|27734229|P81509|CHBB_CROHO CHH-B β subunit | 6 | 8.39 ± 12.2 |
| 10        | gi|118651|P21858|DISI_AGKHA Disintegrin halsyn (platelet aggregation activation inhibitor) | 1 | 1.37 ± 0.15 |
| 11        | gi|13959630|Q91053|VSP1_AAKCA Calobin precursor | 6 | 0.75 ± 0.45 |
| 12        | gi|6093643|P38124|VSP1_BOTJA Platelet-aggregating proteinase PA-BJ | 3 | 0.05 ± 0.02 |
| 13        | gi|13959683|Q9DF67|VSP2_TRIJE Venom serine proteinase 2 precursor (SP2) | 4 | 10.9 ± 9.52 |
| 14        | gi|118655|P18618|DISI_BOTAT Disintegrin batrooxostatin (platelet aggregation activation inhibitor) | 3 | 3.06 ± 2.00 |
| 15        | gi|1171973|P24605|PA22_BOTAS Phospholipase A, homolog 2 (myotoxin II) | 20 | 0.07 ± 0.12 |
| 16        | gi|129400|P20474|PA21_BOTAS Phospholipase A, (myotoxin I) (phosphatidylcholine 2-acylhydrolase) | 9 | 0.12 ± 0.17 |
| 17        | gi|127786|P24330|MYX_CROAD Myotoxin (CAM toxin) | 4 | 0.44 ± 1.09 |
| 18        | gi|170830|P15167|HRTD_CROAT Hemorrhagic metalloprotease HT-D and HT-C precursor (atrolysin D and C) (hemorrhagic toxins C and D) | 11 | >20 |
| 19        | gi|462320|P34182|HRTE_CROAT Hemorrhagic metalloprotease HT-E precursor (atrolysin E) (hemorrhagic toxin E) | 5 | 10.9 ± 7.28 |
| 20        | gi|462318|P20897|HRT2_CROU Hemorrhagic metalloprotease HT-2 (ruberylsin) (hemorrhagic toxin II) | 16 | 15.0 ± 31.1 |
| 21        | gi|461512|P09872|VSP1_AKCO Ancrod (venombin A) (protein C activator) (ACC-C) | 3 | 1.47 ± 0.50 |
| 22        | gi|129507|P00623|PA2_CROAD Phospholipase A, α (phosphatidylcholine 2-acylhydrolase) | 5 | 1.51 ± 0.45 |
| 23        | gi|118660|P17349|DISI_TRIEL Disintegrin elegantin (platelet aggregation activation inhibitor) | 7 | 3.40 ± 1.98 |
| 24        | gi|3914258|P81243|PA21_BOTJA Phospholipase A, (phosphatidylcholine 2-acylhydrolase) (BJ-PLA2) | 7 | <0.05 |
| 25        | gi|17433168|Q9PVE3|PA23_BOTAS Phospholipase A, homolog 3 precursor (myotoxin III) (M1-3-3) | 9 | <0.05 |

*a* Large standard deviation is mainly due to the effect of endogenous proteolytic activities and degenerate peptides.
teinases were solely responsible for the α chain cleavage. However, in 24 h, chains were completely cleaved with both crude and serine proteinase-free venom, and it is obvious that metalloproteinases or other enzymes were also responsible for the α chain cleavage in the serine proteinase-free venom but required a longer time for the cleavage to occur. Type A venom contains serine proteinase that cleaves the α chain but also required more time to do so (Fig. 4, C and D). These serine proteinases are thrombin-like enzymes, which make blood unclottable, and it is safe to state that type A venom contains different serine proteinases affecting either α or β chain because it is unlikely that the same serine proteinases could be affecting both chains (63, 64). A combination of both metallo- and serine proteinases exist in a higher abundance in type B venom than in type A venom (Table II), and thus, the combination of both these proteinases, in particularly the effect of α chain cleavage by metalloproteinases, could very well be the result of the delayed clotting time of blood treated with type B venom (Fig. 5A).

The venoms of B. colombiensis and C. oreganus helleri show very distinct coagulant activities from that of the C. scutulatus scutulatus venoms (Fig. 5). Both venoms have increased procoagulant activity (Fig. 5), but the mechanism of clotting may be due to different venom proteolytic enzymes affecting different factors of the hemostasis pathway (65). B. colombiensis venom cleaves the α and β chains of fibrinogen; C. oreganus helleri also cleaves the α chain and partially cleaves the β chain. The cleavage of the α and β chains by B. colombiensis venom is due to metalloproteinases because when the serine proteinases were removed from the venom cleavage of both α and β chains still occurred in both 2- and 24-h incubation periods (Fig. 4, A, B, C, and D). Again cleav-

![Figure 5](image-url)  
**Fig. 5. Sonoclot graph analysis of venoms on whole human blood.** A glass bead-activated test (gbACT+ kit obtained from Sienco, Inc.) was used to monitor activated clot time, clot rate, and platelet function on a Sonoclot Coagulation and Platelet Function Analyzer (Sienco, Inc.). A, solid line, normal human blood control (no venom added); short dashed line, C. scutulatus scutulatus B; long dashed lines, C. scutulatus scutulatus A. B, solid line, normal human blood control; short dashed line, C. oreganus helleri; long dashed line, B. colombiensis.

| Venoms               | MHDa | MHemDb | Sonoclot ACT, CR, PFc | Fibrinogenolyticd 2 h/24 h | Crude venom | Serine proteinase-free venom |
|----------------------|------|--------|-----------------------|-----------------------------|-------------|-----------------------------|
|                      | µg   | µg     |                       |                             |             |                             |
| C. scutulatus scutulatus A | 162.5 | 9.7    | 144, 10, 2.2          | β/α,β                       |             |                             |
| C. scutulatus scutulatus B | 16   | 4.8    | 354, 6.2, 2.5         | α,β/α,β                     | α/α,β       |                             |
| C. oreganus helleri    | 6    | 4.8    | <26, 51, 1            | α,β/α,β                     | α/α,β       |                             |
| B. colombiensis       | 25   | 2.4    | <26, 51, 1            | α,β/α,β                     | α/α,β       |                             |

| Table IV | Proteolytic and Sonoclot activities of venoms C. scutulatus scutulatus A and B, C. oreganus helleri, and B. colombiensis |
|----------|---------------------------------------------------------------------------------------------------------------------|

* The amount of protein that will cause a 10-mm hemorrhagic spot.  
* Minimal phospholipase A dose: the amount of protein that will cause a 10-mm halo.  
* ACT, activated clot time (s): time in which clot starts forming; CR, clot rate (clot signals/min); PF, platelet function. Normal control range values are: ACT: 128–213; CR: 15–26; PF: 3–5.  
* d α,β, cleavage of α and β chain; α, cleavage of α chain; –, no cleavage.  
* C. scutulatus scutulatus A: neurotoxic venom; C. scutulatus scutulatus B: hemorrhagic venom.
age of the β chain by C. oreganus helleri venom may be due to both metallo- and serine proteinases acting synergistically on it. The hemolytic activity was higher in B. colombiensis than C. oreganus helleri venom; however, C. oreganus helleri venom was more hemorrhagic. The hemolytic activity is due to phospholipases, whereas hemorrhaging is due to metallo- and serine proteinases; by SoPIL analysis phospholipases and metalloproteinases were found in higher abundance in B. colombiensis and C. oreganus helleri venoms, respectively (Table III).

Protease Activity in Snake Venoms—This study also observed for the first time extensive cleavage of venom proteins in all four snake venoms. Tables V and VI illustrate the random protease cleavage of the metalloproteinase jararhagin and metalloproteinase HT-D from the C. scutulatus scutulatus and B. colombiensis/C. oreganus helleri analysis, respectively. A majority of peptides have only one tryptic end (22 of 28 and 13 of 17 in Tables V and VI, respectively), and the cleavage was found on every amino acid residue in some peptides. Such extensive cleavage was most likely the result of a combination of activities by multiple proteases in the snake venom. Proteases and other enzymes in snake venom have multiple purposes such as increasing the prey’s uptake of toxins and the digestion of preys. Such endogenous protease activities in all four venoms could alter the average protein abundance, resulting in a relatively large error in any existing measurement method, including the proteomics method.

It is not clear when venom proteases were activated. The activation might occur during the procedure in which the venoms were collected. After lyophilization and denaturing, the proteases lost their activities. However, it is possible that the proteases were reactivated during the tryptic digestion period, resulting in extensive cleavages of venom proteins. In addition, it is known that trypsin may result in nonspecific cleavages even if sequence grade trypsin was used for better specificity (66).

Conclusion—This study presents the first quantitative proteomics analysis of snake venom from several species based on stable isotope labeling. The new SoPIL reagents that selectively label and isolate cysteine-containing peptides provide a powerful analytical tool to screen snake venoms that are cysteine-rich for many diverse classes of proteins. This new methodology has the advantage of homogeneity of the solution reaction, high efficiency of click chemistry, and convenience of the solid phase capture/release process. The quantitative proteomics study helps us understand geographical and environmental variations in snake venoms; this is crucial in developing better therapeutic agents for treating snakebites. In addition, quantitative proteomics can also be used for discovering new therapeutic molecules present in snake venom. The conclusions of this study support the theory that there are characteristic differences in the venoms within the same species and from different geographical locations (31). It is evident that the SoPIL method can be an efficient tool for snake venom research and for a much wider application of quantitative proteomics.

**Table V**

Partial list of protease cleavage in the venom of C. scutulatus scutulatus A and B for putative venom metalloproteinase jararhagin precursor (HF2 proteinase) (contains disintegrin)

| Peptide sequence<sup>a</sup> | GDNPSVEDHCYRYG | G |
|-------------------------------|-----------------|---|
| P VEDHCYHYG | I |
| G TPNCONECDCAATCK | L |
| N CGNECDAAATCK | K |
| K LKSGQCGHGDCCCEOQCK | F |
| G SGQCGHGDCCCEOQCK | F |
| G SQCGHGDCCCEOQCK | F |
| C GHDCCCEOQCK | F |
| G HGDCCEOQCK | F |
| C RASMSECDDPAEHCTGQSECPADVFHK | N |
| R ASMSECDDPAEHCTGQSECPADVFHK | N |
| R ASMSECDDPAEHCTGQSECPADVFHK | K |
| A SMSECDDPAEHCTGQSECPADVFHK | N |
| M SECEDPAEHCTGQSECPADVFHK | N |
| C DPAEHCTGQSECPADVFHK | N |
| D PAEHCTGQSECPADVFHK | N |
| P ATECDDPAEHCTGQSECPADVFHK | N |
| P ATECDDPAEHCTGQSECPADVFHK | N |
| E HCTGQSECPADVFHK | N |
| T GQSECPADVFHK | N |
| K NGQPCDLNYG | C |
| K NGQPCDLNYG | C |
| K NGQPCDLNYG | C |
| Q KNGYGGCQY | K |
| K GNYGGCQY | K |
| K IPQAPEDVK | C |
| K DNFGQNNPCK | M |
| K VCSNHCDVYATAY | Y |
| K VCSNHCDVYATAY | Y |

<sup>a</sup> **<sup>a</sup>** indicates a tryptic cleavage site; dash indicates a carboxy-terminal residue.

**Table VI**

Partial list of protease cleavage in the venom of B. colombiensis and C. oreganus helleri for hemorrhagic metalloproteinase HT-D and HT-C precursor (atrolysins D and C) (hemorrhagic toxins C and D)

| Peptide sequence* | HNIHMGTMAHELGHNLGMEHDGK | G |
|-------------------|---------------------------|---|
| G KITTNPSVEDHCYRYG | G |
| K ITTNPSVEDHCYRYG | G |
| I TTNPNSVEDHCYRYG | G |
| R GRIENDAGSTASACNGL | G |
| R YIELVYVADHR | V |
| K SHDNAQLLTIAELDEE | T |
| P INLLMGVTMAHELGHNLGMEHDGK | G |
| L MGVTMAHELGHNLGMEHDGK | G |
| M GVMAHELGHNLGMEHDGK | G |
| G VMAHELGHNLGMEHDGK | G |
| T MAHELGHNLGMEHDGK | G |
| A HELGHNLGMEHDGK | G |
| L GHNLGMEHDGK | G |
| N LMGMEHDGK | G |
| G MEHDGK | G |
| R GASLCIMR | G |
| Y KPCQILNKLPR | I |

<sup>a</sup> **<sup>a</sup>** indicates a tryptic cleavage site.
Quantitative Snake Venom Proteomics

Acknowledgments—We thank Professor M. G. Finn at the Scripps Research Institute for the supply of ligand for the click chemistry and for helpful suggestions. We acknowledge the use of software in the Institute for Systems Biology developed using federal funds from the NHLBI, National Institutes of Health, under Contract NO1-HV-28179. We thank Luis Fernando Navarrete for venom extractions done at the Instituto de Medicina Tropical, Universidad Central de Venezuela.

* This work was supported in part by Purdue University, the American Society for Mass Spectrometry, and a National Science Foundation CAREER development award (to W. A. T.). This work was also supported by grants to the Natural Toxins Research Center at Texas A&M University-Kingsville (National Institutes of Health (NIH)/National Center for Research Resources Grant 1 P40 RR018300-01, NIH/Research Infrastructure in Minority Institutions Grant 5 P5000216–02, and NIH/Support of Continuous Research Excellence Grant 5 S06 GM008107-29) and Fondo Nacional de Ciencia, Tecnologia e Innovacion Grant G-2005000400. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

REFERENCES

1. Aebersold, R. (2003) Quantitative proteome analysis: methods and applications. J. Infect. Dis. 187, Suppl. 2, S315–S320
2. Ong, S. E., Foster, L. J., and Mann, M. (2003) Mass spectrometry-based approaches in quantitative proteomics. Methods 29, 124–130
3. Duggan, D. J., Bittner, M., Chen, Y., Meltzer, P., and Trent, J. M. (1999) Expression profiling using cDNA microarrays. Nat. Genet. 21, (suppl.) 10–14
4. Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y., and Aebersold, R. (2000) Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. Proc. Natl. Acad. Sci. U. S. A. 97, 9390–9395
5. Old, W. M., Meyer-Arendt, K., Aveline-Wolf, L., Pierce, K. G., Mendoza, A., Sevinsky, J. R., Resing, K. A., and Ahn, N. G. (2005) Comparison of label-free methods for quantifying human proteins by shotgun proteomics. Mol. Cell. Proteomics 4, 1487–1502
6. Li, X. J., Yi, E. C., Kemp, C. J., Zhang, H., and Aebersold, R. (2005) A software suite for the generation and comparison of peptide arrays from sets of data collected by liquid chromatography-mass spectrometry. Mol. Cell. Proteomics 4, 1328–1340
7. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat. Biotechnol. 17, 994–998
8. Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Hwang, W. M. (2005) Inhibition of adipogenesis by RGD-dependent disintegrin. Annu. Rev. Cell Dev. Biol. 21, 793–826
9. Glenn, J. L., Straight, R. C., Wolfe, M. C., and Hardy, D. L. (1993) Geographical variation in Crotalus scutulatus scutulatus (Mojave rattle snake) venom properties. Toxicon 21, 119–130
10. Glenn, J. L., and Straight, R. C. (1999) Intergradation of two different venom populations of the Mojave rattler (Crotalus scutulatus scutulatus) in Arizona. Toxicon 27, 411–418
11. Sanderson, E. E., Galan, J. A., Powell, R. L., Reyes, S. R., Soto, J. G., Russell, W. K., Russell, D. H., and Perez, J. C. (2005) Disintegrin, hemorrhagic, and proteolytic activities of Mohave rattle snake, Crotalus scutulatus scutulatus venom lacking Mojave toxin. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 141, 124–132
12. Fox, J. W., Ma, L., Nelson, K., Sherman, S. A., and Serrano, S. M. (2006) Comparison of indirect and direct approaches using ion-trap and Fourier transform ion cyclotron resonance mass spectrometry for exploring viper venom proteomes. Toxicon 47, 700–714
13. Sanz, L., Gibbs, H. L., Mackessy, S. P., and Calvete, J. J. (2006) Venom proteomes of closely related Sistrurus rattlesnakes with divergent diets. J. Proteome Res. 5, 2098–2112
14. Birrell, G. W., Earl, S. T., Wallis, T. P., Masci, P. P., de Jersey, J., Gorman, J. J., and Lavin, M. F. (2007) The diversity of bioactive proteins in Australian snake venoms. Mol. Cell. Proteomics 6, 793–806
15. Bandeira, N., Clauser, K. R., and Pevzner, P. A. (2007) Shotgun protein sequencing: assembly of peptide tandem mass spectra from mixtures of modified proteins. Mol. Cell. Proteomics 6, 1123–1134
16. Schwabacher, A. W., Lane, J. W., Schishever, M. W., Leigh, K. M., and Johnson, C. W. (1998) Desymmetrization reactions: efficient preparation of unsymmetrically substituted linker molecules. J. Org. Chem. 63, 1727–1729
17. Kellar, A., Nesvizhskii, A. I., Kolker, E., and Aebersold, R. (2002) Empirical
statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal. Chem. 74, 5383–5392
38. Nesvizhskii, A. I., Keller, A., Kolker, E., and Aebersold, R. (2003) A statistical model for identifying proteins by tandem mass spectrometry. Anal. Chem. 75, 4646–4658
39. Li, X., Zhang, H., Ranish, J. A., and Aebersold, R. (2003) Automated statistical analysis of protein abundance ratios from data generated by stable-isotope dilution and tandem mass spectrometry. Anal. Chem. 75, 6648–6657
40. Omori-Satoh, T., Sadahiro, S., Ohsaka, A., and Murata, R. (1972) Purification and characterization of an antihemorrhagic factor in the serum of Trimeresurus flavoviridis, a crotalid. Biochim. Biophys. Acta 285, 414–426
41. Habermann, E., and Hardt, K. L. (1972) A sensitive and specific plate test for the quantitation of phospholipases. Anal. Biochem. 50, 163–173
42. Glenn, J. L., and Straight, R. (1978) Mojave rattlesnake Crotalus scutulatus scutulatus venom: variation in toxicity with geographical origin. Toxicon 16, 81–84
43. Glenn, J. L., and Straight, R. C. (1982) The rattlesnakes and their venom yield and lethal toxicity, in Rattlesnake Venom: Their Actions and Treatment (Tu, A. T., ed) p. 110, Marcel Dekker, Inc., New York
44. Mosquera, A., Idrovo, L. A., Tafur, A., and Del Brutto, O. H. (2003) Stroke following Bothrops spp. snakebite. Neurology 60, 1577–1580
45. Salazar, A. M., Rodriguez-Acosta, A., Girón, M. E., Aguilar, I., and Guerrero, B. (2007) A comparative analysis of the clotting and fibrinolytic activities of the snake venom (Bothrops atrox) (serpentes:viperidae) from different geographical areas in Venezuela. Thromb. Res. 20, 95–104
46. Rodríguez-Acosta A., Uzcategui, W., Azuaje, R., Aguilar, I., and Girón, M. E. (2000) A clinical and epidemiological analysis of accidental bites by snakes of the genus Bothrops in Venezuela. Rev. Cubana Med. Trop. 52, 90–94
47. Gutierrez, J. M., and Lomonte, B. (1995) Phospholipase A2 myotoxins from Bothrops snake venoms. Toxicon 33, 1405–1424
48. Bober, M. A., Glenn, J. L., Straight, R. C., and Owmby, C. L. (1988) Detection of myotoxin α-like proteins in various snake venoms. Toxicon 26, 665–673
49. Gopalakrishnakone, P., Dempster, D. W., Hawgood, B. J., and Elder, H. Y. (1984) Cellular and mitochondrial changes induced in the structure of murine skeletal muscle by crotoxin, a neurotoxic phospholipase A2 complex. Toxicon 22, 85–98
50. Gralen, N., and Svedberg, T. (1938) The molecular weight of crotoxin. Biochem. J. 32, 1375–1377
51. Metsch, R. B., Dray, A., and Russell, F. E. (1984) Effects of the venom of the Southern Pacific rattlesnake, Crotalus viridis helleri, and its fractions on striated and smooth muscle. Proc. West. Pharmacol. Soc. 27, 395–398
52. Bush, S. P., and Siedenburg, E. (1999) Neurotoxicity associated with suspected southern Pacific rattlesnake (Crotalus viridis helleri) envenomation. Wilderness Environ. Med. 10, 247–249
53. French, W. J., Hayes, W. K., Bush, S. P., Cardwell, M. D., Bader, J. O., and Rael, E. D. (2004) Mojave toxin in venom of Crotalus helleri (Southern Pacific Rattlesnake): molecular and geographic characterization. Toxicon 44, 781–791
54. Sanchez, E. E., Galan, J. A., Perez, J. C., Rodriguez-Acosta, A., Chase, P. B., and Perez, J. C. (2003) The efficacy of two antivenoms against the venom of North American snakes. Toxicon 41, 357–365
55. Ouyang, C., and Teng, C. M. (1976) Fibrinogenolytic enzymes of Trimeresurus mucrosquamatus venom. Biochim. Biophys. Acta 420, 298–308
56. Meier, J., and Stocker, K. (1991) Effects of snake venoms on hemostasis. Crit. Rev. Toxicol. 21, 171–182
57. Braud, S., Bon, C., and Wisner, A. (2000) Snake venom proteins acting on hemostasis. Biochimie (Paris) 82, 851–859
58. Kini, R. M. (2004) Platelet aggregation and exogenous factors from animal sources. Curr. Drug. Targets Cardiovasc. Haematol. Disord. 4, 301–325
59. Markland, F. S. (1998) Snake venoms and the hemostatic system. Toxicon 36, 1749–1800
60. Komalik, F. (1991) The Influence of Snake Venom Proteins on Blood Coagulation, Pergamon Press, New York
61. Kini, R. M., Rao, V. S., and Joseph, J. S. (2002) Procoagulant proteins from snake venoms. Haemostasis 31, 218–224
62. Joseph, J. S., and Kini, R. M. (2004) Snake venom prothrombin activators similar to blood coagulation factor X. Curr. Drug Targets Cardiovasc. Haematol. Disord. 4, 397–416
63. Aronson, D. L. (1976) Comparison of the actions of thrombin and the thrombin-like venom enzymes ancrod and batroxobin. Thromb. Haemostasis 36, 9–13
64. Bell, W. R., Jr. (1997) Delfibrinogenating enzymes. Drugs 54, Suppl. 3, 18–31
65. Kini, R. M., Joseph, J. S., and Rao, V. S. (2002) Prothrombin Activators from Snake Venoms, John Wiley, Chichester, UK
66. Picotti, P., Aebersold, R., and Domon, B. (2007) The implications of proteolytic background for shotgun proteomics. Mol. Cell. Proteomics 6, 1589–1598