SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

DTIC FILE COPY

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
AD-A218 038 DTIC ELECTE 1990 D	3. DISTRIBUTION/AVAILABILITY OF REPORT DISTRIBUTION STATEMENT A Approved for public release Distribution Unlimited
	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION U.S. Army Medical Research Institute of Infectious Diseases 6b. OFFICE SYMBOL (If applicable) SGRD-UIP-B	7a. NAME OF MONITORING ORGANIZATION U.S. Army Medical Research and Development Command
6c. ADDRESS (City, State, and ZIP Code) Fort Detrick, Frederick, MD 21701-5011	7b. ADDRESS (City, State, and ZIP Code) Fort Detrick, Frederick, MD 21701-5011
8a. NAME OF FUNDING/SPONSORING ORGANIZATION 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)

Isolation of a Toxin from Venom of Wagler's Pit Viper Trimeresurus Wagleri

12. PERSONAL AUTHOR(S)

Scott A. Weinstein, Alan W. Bernheimer and Leonard A. Smith

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
	FROM _____ TO _____	31 JAN 90	16

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

A lethal toxin was isolated from Trimeresurus wagleri venom by fast protein liquid chromatography (molecular sieve) and Biorex-70 cation exchange. The toxin had an M_r of approximately 9.5 kD, a pI of 9.6-9.9, did not exhibit subunit behavior in SDS-PAGE profiles and lacked phospholipase A, proteolytic, and hemolytic activity. The toxin had an i.p. LD_{50} of 0.072 mg/kg. The lethal activity was found to represent less than 1% of the total venom protein, which was only 62-65% of crude venom. The toxin lacked antigenic identity with a number of representative neurotoxins and myotoxins. The crude venom shared at least one antigen with Crotalus scutulatus scutulatus venom. This antigen was not Mojave toxin. The toxin appears symptomologically suggestive of a vasoactive peptide or neurotoxin with specific blocking activity upon respiratory innervation.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	
22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code) / 22c. OFFICE SYMBOL

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

ISOLATION OF A TOXIN FROM VENOM OF WAGLERS PIT VIPER, TRIMERESURUS WAGLERI

SCOTT A. WEINSTEIN,[1] ALAN W. BERNHEIMER,[2] AND LEONARD A. SMITH[1*]

[1]Department of Toxinology, Pathology Division, United States Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701-5011, U.S.A.

[2]Department of Microbiology, New York University School of Medicine, 550 First Avenue, New York, NY 10016, U.S.A.

Running Title: Trimeresurus wagleri lethal toxin.

*To Whom Reprints Should Be Requested

S. A. Weinstein, A. W. Bernheimer, and L. A. Smith. Isolation of a toxin from venom of Waglers pit viper Trimeresurus wagleri. *Toxicon* --, -----, 19--. A lethal toxin was isolated from Trimeresurus wagleri venom by fast protein liquid chromatography (molecular sieve) and Biorex-70 cation exchange. The toxin had an M_r of approximately 9.5 kD, a pI of 9.6-9.9, did not exhibit subunit behavior in SDS-PAGE profiles and lacked phospholipase A, proteolytic, and hemolytic activity. The toxin had an i.p. LD_{50} of 0.072 mg/kg. The lethal activity was found to represent less than 1% of the total venom protein, which was only 62-65% of crude venom. The toxin lacked antigenic identity with a number of representative neurotoxins and myotoxins. The crude venom shared at least one antigen with Crotalus scutulatus scutulatus venom. This antigen was not Mojave toxin. The toxin appears symptomologically suggestive of a vasoactive peptide or neurotoxin with specific blocking activity upon respiratory innervation.

INTRODUCTION

Wagler's pit viper Trimeresurus (=Tropidolaemus BRATTSTROM, 1964) wagleri (LEVITON, 1964) is a small to medium-sized (65-70 cm average) arboreal crotaline which ranges throughout Malaysia, the Philippines, Thailand and the Indo-Australian archipelago to Indonesia. T. wagleri secretes one of the more toxic Trimeresurus venoms. A neurological site of action for T. wagleri venom was proposed by SMITH and HINDLE (1931). MINTON (1968) described a component of T. wagleri venom which was dialyzable (smaller than 1kD), thermostable and symptomologically suggestive of a vasoactive peptide. TAN and TAN (1989) used Sephadex G-50 gel filtration and SP-Sephadex C-25 ion exchange to obtain two non-enzymatic toxins. Gel filtration indicated an M_r of 8.9 kD. These workers found that crude wagleri venom did not affect nerve-evoked twitch tension or acetylcholine reduced response of chick biventer muscle, thus suggesting that T. wagleri venom toxins were not neurotoxins. The present study describes fractionation of T. wagleri venom and partial characterization of an isolated lethal toxin.

MATERIALS AND METHODS

Venoms and purified toxins

Trimeresurus wagleri, Naja naja kaouthia and Oxyuranus scutellatus scutellatus venoms were obtained from Ventoxin Laboratories (Frederick, MD). The T. wagleri venom was collected as a pool from three young adult specimens (all females). Individual samples were collected from each specimen and examined separately in order to establish lack of venom protein variation among these snakes. Androctonus australis Hector venom was purchased from Latoxan (Rosans, France).

Crotamine (lot 17) was purchased from Miami Serpentarium Laboratories, Salt Lake City, UT. α–Cobratoxin was purified from venom of Naja naja kaouthia by using Sulphopropyl (SP)-Sephadex chromatography (CHATMAN and DiMARI, 1974). Taipoxin was purified from venom of Oxyuranus s. scutellatus by gel filtration (FOHLMAN et al., 1976). Mojave toxin was purified from venom of Crotalus scutulatus scutulatus (a single adult male) according to the method of WEINSTEIN *et al.* (1985). Toxin II from Androctonus australis Hector venom (AaH II) was purified by successive fractionation on Mono Q, MonoS and Superose-12 column chromatography (Pharmacia, Piscataway, NJ). Purity and identity of crotamine, α–cobratoxin, taipoxin and AaH II were determined by reverse-phase chromatography and amino acid sequence analysis.

Antibodies

Antisera against purified toxins were raised in New Zealand white rabbits using an administration schedule essentially as described by KAISER et al. (1986) with the exception that different toxin dosages were used in the immunization protocol. Crotamine was administered at 10 µg/kg rabbit , α–cobratoxin and AaH II were administered at 6 µg/kg rabbit, and taipoxin was used at 2 µg/kg rabbit. When antibody titers were detected, doses were increased by 25%. IgG was purified from serum using DEAE-Affi-Gel Blue (Bio-Rad, Richmond, CA). Monoclonal antibody to crotoxin was obtained from Dr. John Middlebrook (KAISER and MIDDLEBROOK, 1988). Polyclonal antibody against crude C.scutulatus scutulatus venom and purified mojave toxin was prepared following the immunization schedule of WEINSTEIN *et al.*, (1985).

Lethality determinations

The i.p. LD_{50} of the crude venom was obtained by injection of male Swiss-Webster mice (18-20 g) in five groups of four mice per group. All injections were administered in the lower quadrants of the abdomen. Dosage was derived from a 1 mg/ml solution of venom in phosphate buffered saline (PBS) (0.05 M, pH 7.2). Animals were observed after injection and mortality recorded after 24 hr. The LD_{50} of lethal fractions from FPLC was determined by injecting male Swiss-Webster mice (18-20 g) in five groups which contained four mice per group, while the lethal potency of Bio-Rex 70 fractions was determined using six groups which consisted of two mice per group. Toxin diluent was Tris-HCl (0.05 M, pH 7.2) containing 0.7 M NaCl. In the first two dose levels involving venom fractions, equivalent volumes of buffer were injected into control groups of two mice per group. Animals succumbing to either crude venom or venom fraction dosages were necropsied and any gross tissue pathology examined and noted. The LD_{50} was calculated by the Spearman-Karber method (WORLD HEALTH ORGANIZATION, 1981). The 95% fiducial limits for the LD_{50} were determined.

Determination of protein concentration

Protein concentrations were estimated by the bicinchoninic acid assay (BCA assay, Pierce Chemicals) (SMITH *et al.*, 1985)

Fast Protein Liquid Chromatography (FPLC)

Venom samples were prepared for chromatographic analysis by dissolving 20 mg in 1 ml of 0.05 M Tris HCl, pH 7.2 containing 0.7 M NaCl. Aliquots of 500 ul were centrifuged at

100,000 x g in an Eppendorf 5415 benchtop centrifuge (Eppendorf Laboratories). Initial fractionation of 500 ul of centrifuged venom was performed using an FPLC system (Pharmacia) equipped with a Superose-12 H_r 10/30 (molecular sieve) column. The column was developed with 0.05 M Tris HCl, pH 7.2, containing 0.7 M NaCl. Fractions of 1 ml were collected with a flow rate of 0.5 ml/min. The column eluate was monitored by absorbance at 280 nm and corresponding peak fractions of interest were pooled for further study.

High Performance Liquid Chromatography (HPLC)

Reverse phase chromatography of toxin fractions was carried out in a Bio-Rad Hi-Pore RP-318 column. Solvent A was 12% acetonitrile containing 0.06% trifluoroacetic acid (TFA) and solvent B was 0.05% TFA/70% acetonitrile. Flow rate was 1.0 ml/min and chromatography was performed at 30^{0}C. Column effluent was monitored at 210 nm.

Isoelectric focusing (IEF).

IEF was performed in a 110 ml electrofocusing column (LKB Instruments). The gradient was prepared from a less dense solution consisting of 51 ml of water, 4 ml of 8% (w/v) ampholine (pH 3-10) and a more dense solution consisting of undialyzed sample, 8.5 ml of 8% (w/v) ampholine (pH 3-10) and 25 g of sucrose in a final volume of 55 ml. Focusing was carried out at 4^{0}C for 24 hr with a final potential of 1000 V. Fraction volume was 4 ml.

Bio-Rex 70 cation exchange chromatography.

Superose-12 FPLC fractions containing lethal activity were dialyzed against 0.02 M sodium acetate, pH 6.5, at 4^{0}C. The dialyzed material was then concentrated in an Amicon ultracentrifugation cell (Amicon Laboratories) equipped with a 5,000 dalton exclusion membrane. The concentrated sample (typically 4-6 ml, 2.0-4.0 mg protein) was then applied to a 10 x 0.5 cm Econo-column (Bio-Rad Laboratories) packed with Bio-Rex 70 resin (Bio-Rad Laboratories). The column was run at 4^{0}C with a flow rate of 32 ml/hr and fractions of 2.5 ml were collected. After three bed volumes had passed through the column, a 150 ml linear gradient was initiated with 1.2 M NaCl in 0.02 M sodium acetate, pH 6.5. The column eluate was monitored at 280 nm and fractions of high absorbance were examined for lethality and purity.

Determination of proteolytic activity

Presence of proteolytic activity was determined by use of a casein agarose gel substrate (Bio-Rad Laboratories). Venom solutions from 0.125 mg/ml to 1 mg/ml were added to wells

punched in the gel plate, and zones of clearing were measured after 20 hr at 25°C. A 1 mg/ml trypsin solution similarly prepared was used as a standard. PBS (0.05 M, pH 7.2) was used as diluent for venom and typsin solutions. A well filled with PBS served as a negative control.

ELISA

Enzyme-linked immunosorbent assays (ELISA) were carried out essentially as described by HENDERSON and BIEBER (1986). Peroxidase-conjugated, goat anti-rabbit IgG (Sigma) served as secondary antibody when the primary antibody used was rabbit polyclonal. When the primary antibodies were monoclonal, peroxidase-conjugated, rabbit anti-mouse IgG (Kirkegaard and Perry Labs., Gaithersburg, MD) was used as the secondary antibody.

Sodium dodecyl sulfate -Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a Protean II mini-gel apparatus (Bio-Rad Laboratories) using a 12.5 % gel according to the protocol of LAEMMLI (1970). The gel was run at 100V, 25 mA for 65 min, then was stained with either silver nitrate or coomassie blue R-250.

Phospholipase A and hemolytic assays

Qualitative analysis for phospholipase A (PLA) activity was performed following the protocol of MARINETTI (1965). Hemolytic activity was assayed by using rat erythrocytes accoding to the method of BERNHEIMER *et al.* (1976).

RESULTS

Trimeresurus wagleri crude venom lethal potency, proteolytic and hemolytic activity

Crude venom of T. wagleri was found to possess moderate proteolytic activity (Table I). Crude venom protein content was 62-65%. The murine i.p. LD_{50} of crude venom pooled from three snakes was 4.36 mg/kg. Mice succumbing to the effects of crude venom showed a sudden onset of tachypnea and tremors which led to rapid prostration and cardiorespiratory death. Gross necropsy indicated some pulmonary congestion with no other apparent gross effects. The crude venom in concentrations up to 500 µg/ml lacked direct hemolytic activity.

Isoelectric focusing of T. wagleri venom

Isoelectric focusing of crude T. wagleri venom resulted in four major peaks (Fig.1). Strong PLA activity was detected in fraction 24 (pI = 8.9) while weak activity was found in fraction 21 (pI = 7.6). Lethal activity was detected in fractions 26-30 (pI=9.6-9.9).

FPLC analysis of T. wagleri venom

FPLC with cation and anion exchange or hydrophobic resin was unsuccessful in isolating the material responsible for lethal activity that consistently appeared as an unbound peak in the void volume. Fig. 2 shows the profile obtained from Superose-12 (molecular sieve) FPLC analysis of T. wagleri venom. Approximately nine peaks were observed. Lethal activity was detected only in peak D (fractions 18-19). SDS-PAGE indicated a single band with an M_r of about 12.5 kD and a deeply staining smear which had a leading edge of 9.5 kD (Fig. 3). The lethal potency of peak D was 0.9 mg/kg, indicating a five fold increase in specific activity (Table I). Necropsy of mice succumbing to the lethal pool revealed no difference from those which succumbed to the crude venom. The lethal pool contained low levels of proteolytic activity.

Antigenic identity of Superose 12 lethal pool components

Immunological survey by indirect ELISA of both crude T. wagleri and Superose-12 peak D indicated a lack of antigenic identity between wagleri venom toxins and alpha-cobratoxin from Naja n. kaouthia, taipoxin from Oxyuranus scutellatus, crotoxin and crotamine from C. durrissus terrificus, and Mojave toxin from C. scutulatus scutulatus. A moderate reaction of identity was noted with polyclonal antibody against crude "type A" Crotalus scutulatus scutulatus venom. ELISA detection of extensive sharing of antigens among crotaline venoms has been noted by MINTON et al. (1984). Ouchterlony double immunodiffusion of peak D resulted in data identical to those obtained with ELISA. Reaction of the lethal pool with anti-Crotalus scutulatus scutulatus crude venom antiserum resulted in a single precipitin band. Reaction of the lethal pool with anti-Mojave toxin antiserum was negative.

Phospholipase A activity of Superose-12 peak D

A qualitative assay for phospholipase A activity (MARINETTI, 1965) detected weak PLA activity in the Superose 12 lethal pool.

Bio-Rex 70 isolation of lethal toxin from Superose-12 pool

Application onto a Bio-Rex 70 (cation exchange) column of Superose-12 peak D resolved the lethal pool into at least five peaks (Fig.4). Fractions 51-55 contained all of the lethal

activity, which comprised less than 1% of total venom protein (Table I). Fraction #51 exhibited the highest specific toxicity (data not shown). The lethal potency of these fractions was found to be 72 µg/kg (Table I), placing the lethal index of purified wagleri toxin in the range of the highly toxic crotaline polypeptides, crotoxin and Mojave toxin. SDS-PAGE indicated a deeply staining smear with an Mr of approximately 9.5 kD (Fig.3). The toxin did not exhibit subunit behavior when analyzed by SDS-PAGE in the presence of reducing and non-reducing conditions. The toxin lacked proteolytic, PLA and hemolytic activities.

Reverse Phase FPLC analysis of Superose-12 lethal peak D and Bio-Rex 70 lethal toxin

Reverse phase FPLC of Superose-12 peak D indicated that the lethal pool contained five to six components (Fig.5, Panel A). Analysis of toxic fractions (#51 - #55) obtained from Bio-Rex 70 fractionation of peak D from Superose 12 indicated the presence of two major components (Fig.5, Panel B).

DISCUSSION

SMITH and HINDLE (1931) described T. wagleri venom as neurotoxic. MINTON (1968) described a lack of rear limb paresis in mice injected with T. wagleri venom. TAN and TAN (1989) found that T. wagleri venom did not have a curaremimetic effect upon chick biventer cervicis nerve-muscle preparations. These workers also noted a lack of hemorrhagic and necrotizing activity.

The toxin isolated in the present study did not cause paresis in mice but did elicit tachypnea, rapid collapse and spasms. The crude venom exhibited identical symptomology. Necropsy of animals succumbing after 12-20 hr to venom or toxin injections indicated a lack of gross hemorrhage or myonecrosis. The lack of apparent neurotoxic activity is in agreement with the observations of MINTON (1968) and TAN and TAN (1989). The isolated toxin had an approximate M_r of 9.5 kD, a pI of 9.6-9.9, and lacked PLA and hemolytic activity. The toxin was not a protease. The non-enzymatic nature of the toxin described presently agrees with the data of TAN and TAN (1989); however, these workers reported isolation of two toxins, both with M_r of 8.9 kD and different lethal potencies (0.170 mg/kg and 0.190 mg/kg, respectively). The lethal potency of purified toxin (0.072 mg/kg) obtained in the present study is comparable with the two most toxic fractions isolated previously from crotaline venoms crotoxin (0.060 mg/kg-i.p., HENDON and FRAENKEL-CONRAT, 1971) and Mojave toxin (0.094mg/kg-i.p. WEINSTEIN et al., 1985). We feel it is important to note, however, that due to scarcity of crude venom (and subsequently, venom

fractions) in the present study, small groups of mice were used in purified toxin lethality determinations. This may have introduced some error into the reported LD_{50} value.

The toxin caused symptomology suggestive of a hypotensive peptide or a toxin with a highly specific blockade action upon respiratory innervation. Hypotensive peptides such as those isolated from C. viridis helleri (DUBNOFF and RUSSELL, 1970; SCHAEFFER et al., 1979) and C. atrox (BONILLA and RAMMEL, 1976) cause rapid collapse, shock and respiratory arrest leading to death. These toxins and several myotoxins (most notably myotoxin a - OWNBY et al., 1976) have basic pIs in the range of 9-10 (CAMERON and TU, 1977,1978; OWNBY, 1982).

The unique morphological status among the genus Trimeresurus of T. wagleri was noted by BRATTSTROM (1964). MINTON (1974) commented on the distinct nature of T. wagleri venom compared with that of other Trimeresurus venoms. When compared with other Trimeresurus venom toxins, the lethal toxins of T. wagleri also appear unusual. SUGIHARA et al. (1983) described a lethal hemorrhagic toxin designated "mucrotoxin A" from the venom of T. mucrosquamatus. NIKAI et al. (1985) confirmed the hemorrhagic action of mucrotoxin A and isolated an additional hemorrhagic toxin ("mucrotoxin b"). These hemorrhagins had M_r of 15 kD and 27 kD, respectively. OMORI-SATOH and SADAHIRO (1979) resolved a lethal hemorrhagin of T. flavoviridis into two 60 kD components. Most recently, SEKOGUCHI et al. (1989) characterized two lethal toxins (designated "TT-1" and "TT-2") from venom of T. tokarensis. Toxin TT-1 had an M_r of 71 kD, a high content of aspartic acid and glutamic acid, and was hemorrhagic as well as proteolytic. Toxin TT-2 had an M_r of 25.4 kD, high content of glutamic acid and serine, and was lethal. TT-2 was proteolytic, but not hemorrhagic. Both toxins were necrotizing and had LD_{50} values of 6.6 ug/g and 7.2 ug/g, respectively. These toxins required divalent cations (Ca^{2+} and Zn^{2+}) for activity and were thermolabile. From these data, it is clear that T. wagleri venom toxins can be considered aberrant in their lack of hemorrhagic activity and absence of metalloproteolytic lethal components and necrotic activity. The high lethal index of T. wagleri venom toxin appears distinct and could hypothetically be related to an absence of high concentrations of protease, which are significantly higher in other Trimeresurus. Most crotaline venoms with a high lethal index exhibit little, if any, proteolytic activity (GLENN et al., 1983; MINTON and WEINSTEIN, 1984). The pI (9.6-9.9) of T. wagleri lethal toxin is compatible with the non-binding behavior observed upon toxin application to anion exchange and hydrophobic resin.

The semi-purified toxin lacked antigenic identity with a selection considered fairly representative of ophidian venom neurotoxins and myotoxins derived from elapine elapid

and crotaline viperid venoms. Crude T. wagleri venom was found to contain at least one antigen in common with Crotalus scutulatus scutulatus crude venom. This antigen was not Mojave toxin. Weinstein et al. (1985) found no immunoidentity between Mojave toxin and crude venom of T. wagleri. Minton (1968) found that antiserum against venom of T. flavoviridis failed to neutralize T. wagleri venom, even though up to six antigens in T. wagleri venom were shared with T. flavoviridis, T. stejnegeri, and T. mucrosquamatus. These data suggest that T. wagleri lethal venom components are antigenically unique.

Acknowlegements: Drs. Sherman A. Minton and Jim Schmidt provided valuable discussion. The excellent technical assistance of Clem DeWitt and Young Choi is gratefully acknowledged.

REFERENCES

BERNHEIMER, A. W., AVIGAD, L. S. and AVIGAD, G. (1975) Interactions between aerolysin, erythrocytes, and erythrocyte membranes. *Infect. Immun.* **11**, 1312-1319.

BONILLA, C. A., and RAMMEL, O. J. (1976) Comparative biochemistry and pharmacology of salivary gland secretions. III. Chromatographic isolation of a myocardial depressor protein (MDP) from the venom of Crotalus atrox. *J. Chromatogr.* **124**, 303-314.

BRATTSTROM, B. H. (1964). Evolution of the pit vipers. *Trans. San Diego Soc. Nat. Hist.* **13**, 185-268.

CAMERON, D. L., and TU, A. T. (1977). Characterization of myotoxin a from the venom of prairie rattlesnake (Crotalus viridis viridis). *Biochemistry* **16**, 2546-2553.

CAMERON, D. L., and TU, A.T. (1978). Chemical and functional homology of myotoxin a from prarie rattlesnake venom and crotamine from South American rattlesnake venom. *Biochimie Biophys. Acta* . **532**, 147-154.

CHATMAN, V. B. and DiMARI, S. J. (1974) Use of sulphopropyl-sephadex chromatography and disc gel electrophoresis for the fractionation and identification of elapid venoms. *Toxicon* **12**, 405-414.

DUBNOFF, J. W. and RUSSELL, F. E. (1970). Isolation of lethal protein and peptide from Crotalus viridis helleri venom. *Proc. West Pharm. Soc.* **13**, 98.

FOHLMAN, J. EAKER, D., KARLSSON, E. and THESLEFF, S. (1976) Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the Australian

snake Taipan (Oxyuranus scutellatus). *Eur. J. Biochem.* **68**, 457-469.

GLENN, J. L., and STRAIGHT, R. C. (1982). The rattlesnakes and their venom yield and lethal toxicity. In: Rattlesnake Venoms: Their Action and Treatment. (TU, A. T., Ed.) Marcel Dekker (N.Y.).

HENDERSON, J. T. and BIEBER, A. L. (1986) Antigenic relationships between mojave toxin subunits, mojave toxin and some crotalid venoms. *Toxicon* **24**, 473-479.

HENDON, R. A., and FRAENKEL-CONRAT, H. (1971). Biological roles of the two components of crotoxin. *Proc. Nat. Acad. Sci. U.S.A.* **68**, 1560-1563.

KAISER, I. I., MIDDLEBROOK, J. L., CRUMRINE,M. H., and STEVENSON, W. W. (1986) Cross-reactivity and neutralization by rabbit antisera raised against crotoxin, its subunits and two related toxins. *Toxicon* **24**, 669-678.

KAISER, I. I. and MIDDLEBROOK, J. L. (1988) Preparation of a crotoxin neutralizing monoclonal antibody. *Toxicon* **26**, 855-865.

LAEMMLI, V. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

MARINETTI, G. V. (1965). The action of phospholipase A on lipoproteins. *Biochimie Biophys. Acta* . **98**, 554-565.

MINTON, S. A. (1968). Preliminary observations on the venom of Wagler's pit viper (Trimeresurus wagleri). *Toxicon* **6**, 93-97.

MINTON, S. A. (1974). Venom Diseases. Thomas (Springfield).

MINTON, S. A., and WEINSTEIN, S. A. (1984). Protease activity and lethal toxicity of venoms from some little known rattlesnakes. *Toxicon* **22**, 828-830.

MINTON, S. A., WEINSTEIN, S. A., and WILDE, C. E. (1984). An enzyme- linked immunoassay for detection of North American pit viper venoms. *J. Toxicol. Clin .Toxicol.* **22**, 303-316.

NIKAI, T., MORI, N., KISHIDA, M., KATO, Y., TAKENAKA, C., MURAKAMI, T., SHIGEYANE, S., and SUGIHARA, H., (1985). Isolation and characterization of hemorrhagic factors a and b from the venom of the Chinese habu snake (Trimeresurus mucrosquamatus). *Biochimie Biophys. Acta.* **838**, 122-131.

OMORI-SATOH, T., and SADAHIRO, S. (1979). Resolution of the major hemorrhagic component of Trimeresurus flavoviridis venom into two parts. *Biochimie Biophys. Acta* . **580**, 392-404.

OWNBY, C. L., CAMERON, D., and TU, A. T. (1976). Isolation of myotoxic component from rattlesnake (Crotalus viridis viridis) venom. *Am. J. Pathol.* **85**, 149-157.

OWNBY, C.L. (1982). Pathology of rattlesnake envenomation. In: Rattlesnake venoms: Their Actions and Treatment. (A.T. TU, ed.). Marcel Dekker (N.Y.).

SEKOGUCHI, S., NIKAI, T., ITOH, M., and SUGIHARA, H. (1989). Two toxins from the venom of Trimeresurus tokarensis. *Int. J. Biochem.* **21**, 279-289.

SMITH, M. A., and HINDLE, E. (1931). Experiments with the venom of Laticauda, Pseudechis and Trimeresurus species. *Trans. Royal Soc. Trop. Med.*

Hyg. **25**, 115-120.

SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. M., OLSON, B. J., and KLENK, D.C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.

SUGIHARA, H., MORIURA, M., and NIKAI, T. (1983). Purification and properties of a lethal, hemorrhagic protein, "Mucrotoxin A" from the venom of the Chinese habu snake (Trimeresurus mucrosquamatus). *Toxicon* **21**, 247-255.

TAN, N. H., and TAN, C. S. (1989). The enzymatic activities and lethal toxins of Trimeresurus wagleri (speckled pit viper) venom. *Toxicon* **27**, 349-357.

WEINSTEIN, S. A., MINTON, S. A., and WILDE, C. E. (1985). The distribution among ophidian venoms of a toxin isolated from venom of the mojave rattlesnake (Crotalus scutulatus scutulatus). *Toxicon* **23**, 825-844.

WORLD HEALTH ORGANIZATION (1981). Progress in the characterization of venoms and standardization of antivenoms. *WHO Offset Publi.* 58, 23-24.

LEGENDS FOR FIGURES

FIG. 1. ISOELECTRIC FOCUSING OF CRUDE VENOM OF T.wagleri.
Isoelectric focusing was performed in a 110 ml electrofocusing column. The gradient was prepared from a less dense solution consisting of 51 ml of water, 4 ml of 8% (w/v) ampholine (pH 3-10) and a more dense solution consisting of undialyzed sample, 8.5 ml of 8% (w/v) ampholine (pH 3-10) and 25 g of sucrose in a final volume of 55 ml. Focusing was carried out at 4°C for 24 hr with a final potential of 1000 V. Fraction volume was 4 ml.

FIG.2. FPLC MOLECULAR SIEVE CHROMATOGRAPHY OF CRUDE T.wagleri VENOM.
Molecular sieve chromatography was performed by using a Superose 12 HR10/30 column attached to the FPLC unit. Elution was carried out with Tris HCl 0.05 M, pH 7.2, containing 0.7 M NaCl. A flow rate of 0.5 ml/min was maintained and fractions of 1.0 ml were collected. All fractions were monitored for absorbance at 280 nm and lethal activity.

FIG. 3. SDS-PAGE OF T.wagleri VENOM FRACTIONS.
Polyacrylamide gels (12.5%) were cast and run with a Bio-Rad mini Protean II unit according to the method of Laemmli (1970). Apparent molecular weights were determined by Andrews plots. Proteins were detected by staining with silver nitrate. Lane 1 - Bio-Rex 70 fraction #51; lane 2 - Superose 12, peak D; and lane 3 - molecular weight markers: phosphorylase b = 94,000, bovine serum albumin = 67,000, ovalbumin = 43,000, carbonic anhydrase = 30,000, soybean trypsin inhibitor = 20,100, α-lactalbumin = 14,400.

FIG.4. BIO-REX 70 CATION EXCHANGE CHROMATOGRAPHY OF MOLECULAR SIEVE (SUPEROSE 12) LETHAL PEAK D POOL.
Lethal fractions obtained by molecular sieve chromatography were pooled and dialyzed against 0.02 M sodium acetate, pH 6.5, at 4°C. The dialyzed material

was then concentrated in an Amicon ultrafiltration cell(Amicon Laboratories) equipped with a 5,000 Dalton exclusion membrane. The concentrated sample was then applied to a 10 x 0.5 Econo-column (Bio-Rad Laboratories) packed with Bio-Rex 70 resin (Bio-Rad Laboratories). The column was run at 4°C with a flow rate of 32 ml/hr and fractions of 2.5 ml were collected. After three bed volumes had passed through the column, a 150 ml gradient was initiated with 1.2 M NaCl in 0.02 M sodium acetate, pH 6.5. The column eluate was monitored at 280 nm and fractions of high absorbance were examined for lethality and purity.

FIG.5. REVERSE PHASE(HPLC) ANALYSIS OF LETHAL FRACTION POOLS OBTAINED BY MOLECULAR SIEVE (SUPEROSE 12) AND CATION EXCHANGE (BIO-REX 70) CHROMATOGRAPHY.

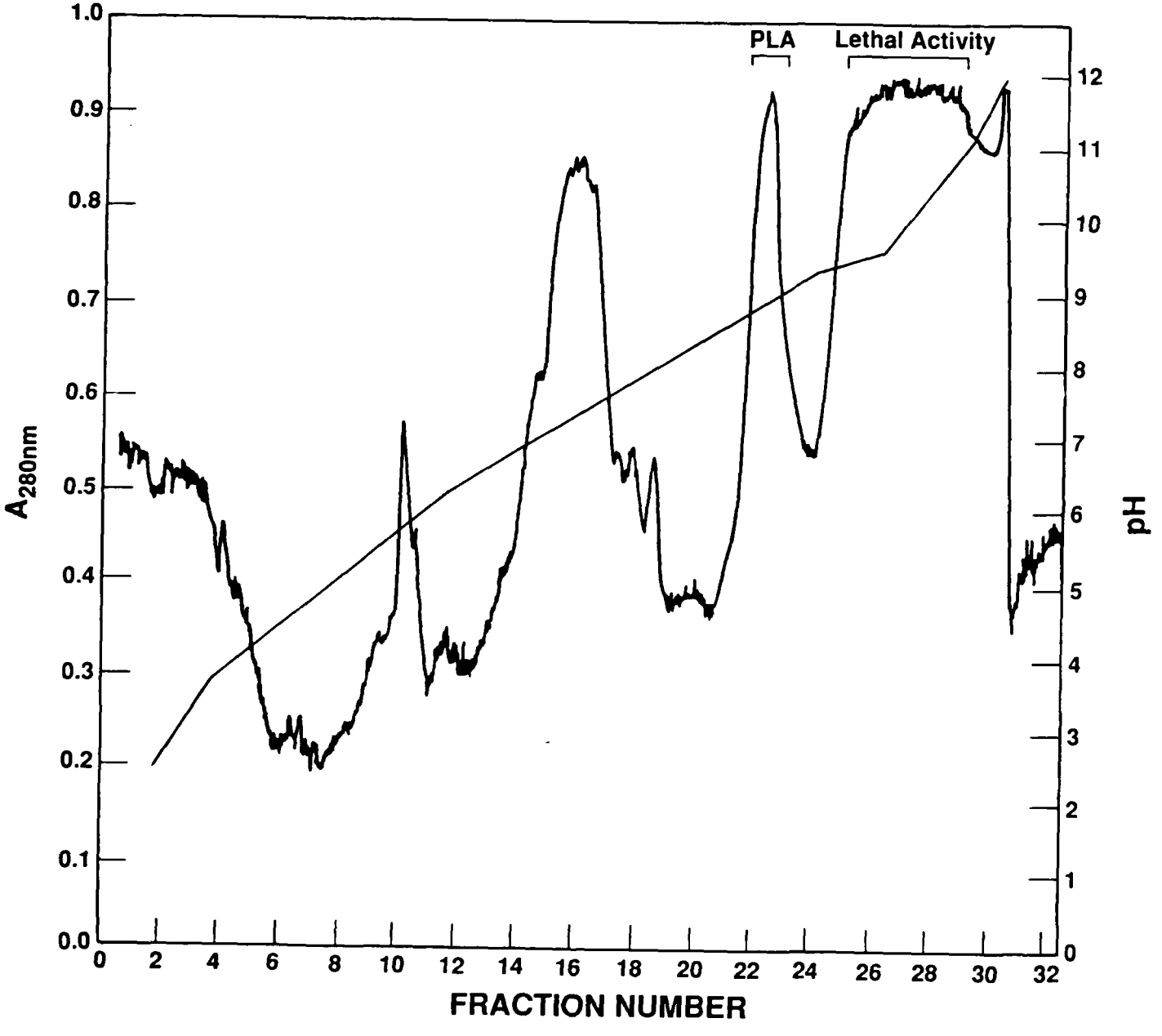
Reverse phase chromatography of toxin fractions was carried out in a Bio-Rad Hi-Pore RP-318 column. Solvent A was 12% acetonitrile containing 0.06% trifluoroacetic acid (TFA) and solvent B was 0.05% TFA/70% acetonitrile. Flow rate was 1.0 ml/min and chromatography was performed at 30°C. Column effluent was monitored at 210 nm. Panel A is the HPLC profile of Superose 12 lethal peak D and panel B is Bio-Rex 70 fraction # 51.

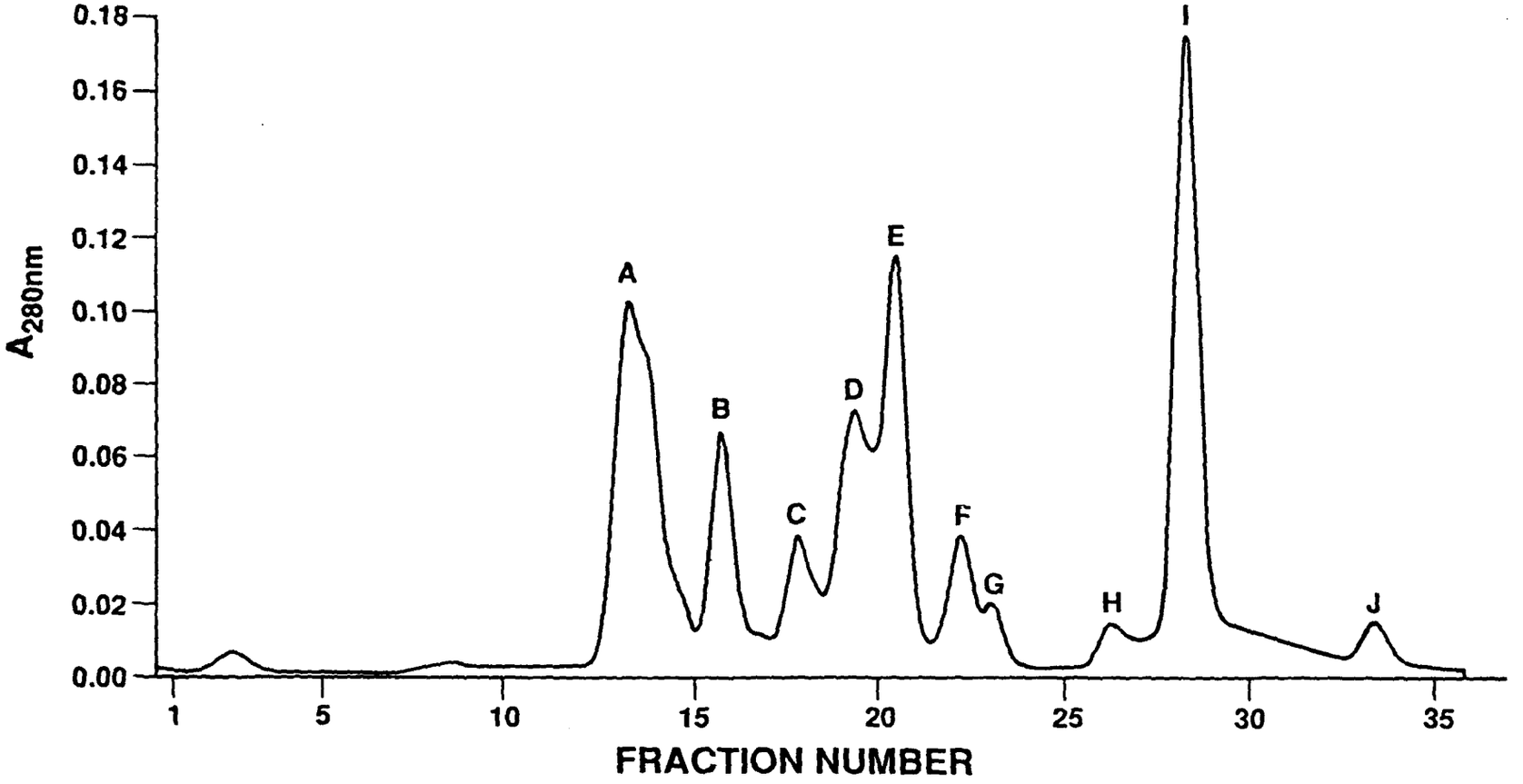
Table 1. Purification, Activity And Recovery Of Trimeresurus wagleri Lethal Activity

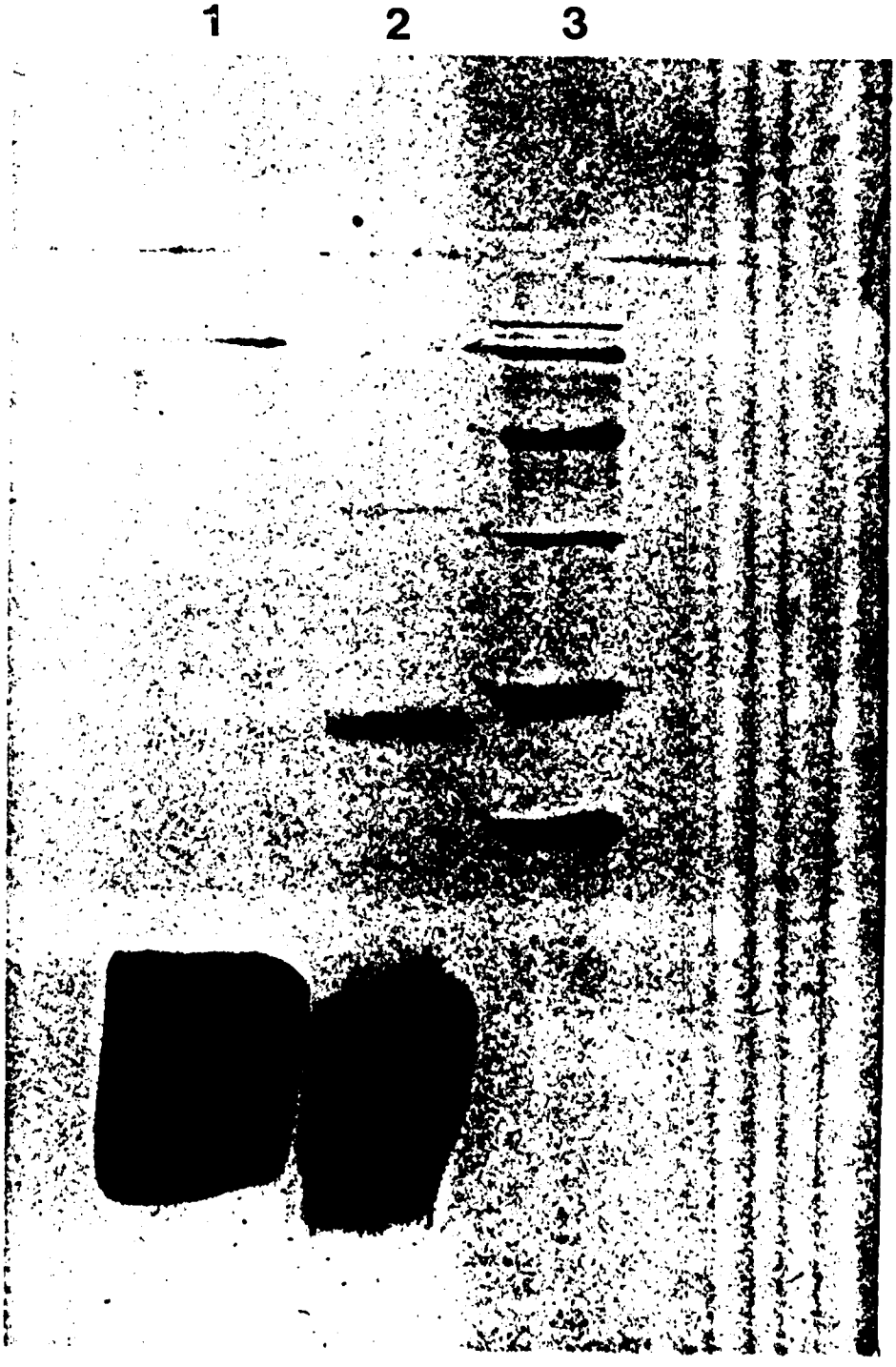
Sample	Protein (mg)	Total Volume	Total Protein	Murine i.p. Lethality (LD_{50})	[1]Specific Activity	% Protein Recovery	[2]Protease Activity
Crude venom	10.05 mg / 0.5 ml	2.5 ml	50.2 mg	4.36 mg / kg	0.23	100 %	35
Concentrated Superose-12 Pool	0.497 mg / ml	4.5 ml	2.2 mg	0.90 mg / kg	1	4.30 %	17
Concentrated Bio-Rex 70 Pool	0.030 mg / ml	11 ml	0.330 mg	0.072 mg / kg	13.8	0.65 %	0

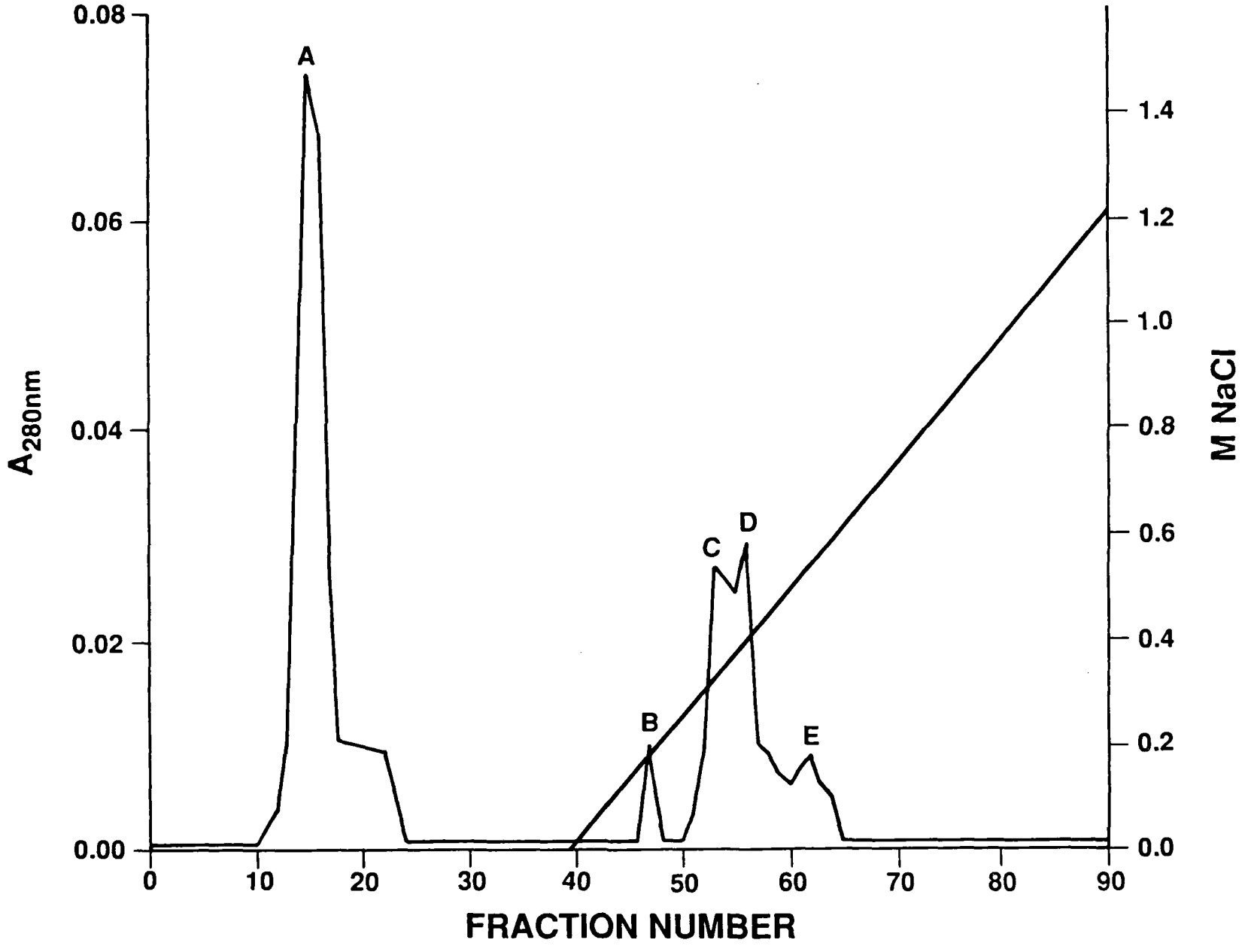
[1] Lethality / protein = 11.5 LD_{50} / 50.2 mg crude venom = specific activity of 0.23

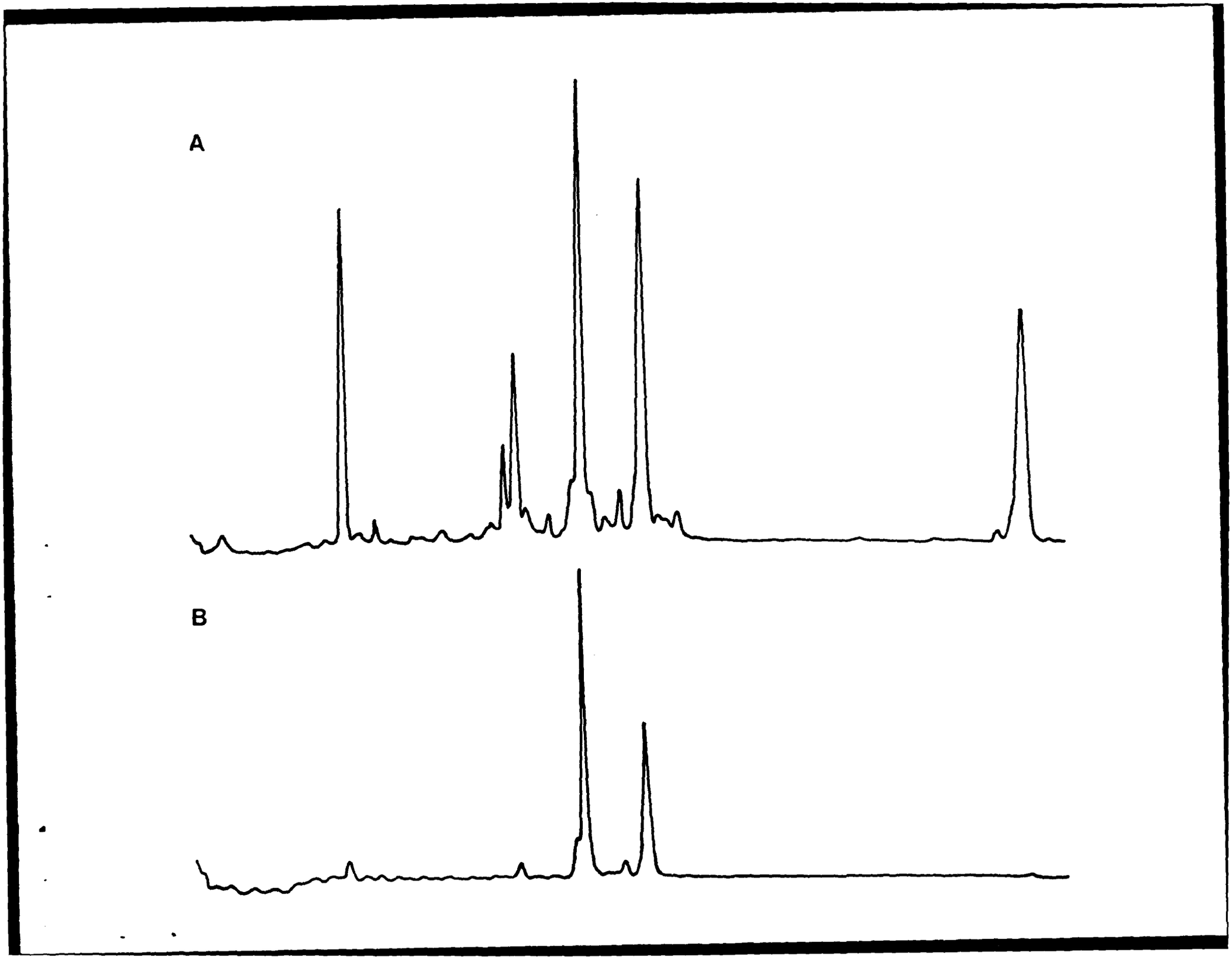
[2] Units of trypsin caseinolytic activity per mg of crude venom or fraction protein


PLA
Lethal Activity
A_{280nm}
pH
FRACTION NUMBER
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
12
11
10
9
8
7
6
5
4
3
2
1
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32


0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
A_{280nm}
A
B
C
D
E
F
G
H
I
J
1
5
10
15
20
25
30
35
FRACTION NUMBER


1
2
3


0.08
0.06
0.04
0.02
0.00
A
B
C
D
E
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
A280nm
M NaCl
FRACTION NUMBER


A
B