STUDIES ON THE HEMORRHAGIC TOXIN, PROTEINASE H, FROM *CROTALUS ADAMANTEUS* (EASTERN DIAMONDBACK RATTLESNAKE) VENOM

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Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1991

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 1996

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ACKNOWLEDGEMENTS

I thank God and our Lord and Savior Jesus for enabling me to complete this work and for His guidance during this period of my life.

I wish to thank Dr. Charlotte L. Ownby as my dissertation adviser and an excellent mentor for her unerring guidance and unending support and for allowing me just enough rope to hang myself with (and then stopping me just before I did). I also thank Dr. James Blankemeyer, Dr. James Breazile, Dr. Joseph McCann and Dr. Larry Stein for serving on my committee. Additionally, I'd like to thank Terry Colberg for his inestimable guidance, help and advice in the lab.

I would also like to express my sincere gratitude to all those persons affiliated with the Department of Physiological Sciences for making this department such a comfortable and rewarding place in which to study. I am also very grateful to the Ford Foundation and the OSU Foundation for providing the funding which made this project possible.

Finally, thanks to my loving wife, Tammie, for being my friend, confidant, and constant support throughout this work and for lending her special, considerate touch to making this experience not only intellectually rewarding but also relaxed and fun. I would also like to thank my parents and family for their love, support, help, and patience. They have been my inspiration and their love and nurturing made this all possible.

I wish to dedicate this work to the memory of my grandfather, Josh Norwood.

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CHAPTER I

INTRODUCTION

Medical Importance of Venomous Snakes

Humanity's relationship with serpents throughout recorded history has been a tenuous one at best. The snake has long been the subject of fear as well and reverence. In more recent decades, as human knowledge and understanding of nature have continued to increase, we have learned that which makes some snakes fearsome, their venoms, can not only be combated but also harnessed as useful tools for the betterment of humanity. Snake venoms merit a great deal of attention as in many parts of the world they represent serious human health concerns. Approximately 15 % of the 3000 known species of snakes are venomous. Venomous snakes are classified into the families Viperidae, Elapidae, Hydrophidae and Colubridae (MEHRTENS, 1987). In the United States only envenomation by members of the families Elapidae (coral snake) and Viperidae (copperheads, rattlesnakes, cottonmouths) produce medical problems. Snakebite envenomations can induce a variety of systemic pathologies including cardiovascular and respiratory dysfunctions, hypotension, neurological disorders, and coagulopathies (GOMEZ and DART, 1995). Lethality is usually attributed to respiratory failure and shock (GOMEZ and DART, 1995). It has been estimated that approximately 8000 poisonous snake bites occur in the U. S. annually (PARRISH, 1966) with less than 0.5 % of these being fatal (PARRISH, 1966; RUSSELL, 1983). This is a great improvement over the fatality

rate of 5-25 % seen at the beginning of the twentieth century (GOMEZ and DART, 1995). Polyvalent (Crotalidae) antivenin (Wyeth) is currently used almost exclusively in U. S. hospitals for the treatment of snakebite envenomation. The low mortality rate of poisonous snake bites may be at least partially attributable to the use of this product (RUSSELL, 1983). Snake venoms also elicit serious damage to local tissues. These local effects of snake venoms include myonecrosis, edema, and hemorrhage (OWNBY, 1982). If unchecked these effects may result in permanent tissue loss, severe disfigurement, immobilization, and even the necessity of amputation (GOMEZ and DART, 1995). Thus, while the threat of death from poisonous snake bites may not be particularly alarming, the maiming, scarring, and debilitation due to the effects of snake venom on local tissues can be quite serious.

Venom-induced hemorrhage

Upon gross observation snake venom-induced hemorrhage may present itself as either diffuse petechiation or ecchymosis surrounding the point of envenomation (GOMEZ and DART, 1995). Hemorrhage not only has a direct effect on tissues, but also can produce indirect effects due to ischemia by simple loss of vascularization to a tissue or shifts in the tissue distribution of circulating blood volume to compensate for blood lost in hemorrhage (PRICE *et al.*, 1993). In very severe cases, blood loss due to local and systemic hemorrhage may make a significant contribution to the lethality of a venom or may exacerbate circumstances in a synergistic fashion with the effects of other venom components (KAMIGUTI *et al.*, 1992).

Hemorrhagic toxins

It is now well-established that venom-induced hemorrhage is primarily the result of hemorrhagic metalloproteinases (MEIER and STOCKER, 1995). To date there have been approximately 80 snake venom hemorrhagic toxins isolated and characterized (Table 1). Snake venom hemorrhagic toxins are zinc-dependent metalloproteinases ranging in size from 15 to 100 kD and having activity against a wide variety of substrates (MARSH, 1994; BJARNASON and FOX, 1994) (Table 1). Studies on the structural and functional similarities of hemorrhagic toxins have revealed two structural features which are thought to be common to all. Firstly, sequence analysis of several snake venom hemorrhagic toxins revealed the presence of an amino acid motif characteristic of zinc-dependent metallopeptidases (JONGENEEL *et al.*, 1989; JIANG and BOND, 1992). This distinguishing signature of snake venom metalloproteinases is formed by a conserved amino acid consensus sequence of HEBXHXBXXHX ("B" = bulky, apolar residue; "E" = glutamic acid; "H" = histidine; "X" = variable residue) containing the active zinc-binding site (KINI and EVANS, 1992; BJARNASON and FOX, 1994). Secondly, snake venom metalloproteinases, including hemorrhagic toxins, also have a highly conserved methionine-containing turn (met-turn) in which methionine is contained in the sequence CIMXP ("C" = cysteine; "I" = isoleucine; "M" = methionine; "X" = variable residue; "P" = proline) (BJARNASON and FOX, 1994). Snake venom hemorrhagic toxins have thus been classified as belonging to the reprolysin family of zinc-metalloproteinases, a family within the metzincin superfamily of enzymes. This family also includes certain mammalian reproductive enzymes (HOOPER, 1994) such as PH-30a, PH-30b, EAP I, and Cyritestin (WOLFSBERG *et al.*, 1993).

TABLE 1. CHARACTERISTICS OF KNOWN HEMORRHAGIC TOXINS ISOLATED FROM SNAKE VENOM

Venom	Toxin	MW (Kd)	pI	Substrates	MHD (μg)	Effect on Fg	+CHO	Inhibitors	Ref.
Agkistrodon acutus									
	AC_1-protease	24.5	4.7	casein, insulin B, Fg, azocasein, HPA, azoalbumin	0.22	α	N. D.	EDTA, cys.	NIKAI *et al.*, 1977
	AC_2-protease	25	4.9	casein	0.43			EDTA, cys.	SUGIHARA*et al.*, 1978
	AC_3-protease	57	4.7	Fg	0.95	α & β	0.1%	EDTA, cys.	YAGAHASHI *et al.*, 1986
	AC_4-protease	33	4.4	casein	0.31			EDTA, cys.	SUGIHARA*et al.*, 1980
	AC_5-protease	24	6.7	casein	0.37			EDTA, cys.	MORI *et al.*, 1984
	FP	24	3.8	Fg	3.81	α		EDTA, cys.	OUYANG and HUANG, 1976
	A_a-toxin I	22	4.6	casein	0.4-2.5			EDTA, cys.	XU *et al.*, 1981
	A_a-toxin II	22	5.3	fibrin?	1.5			cys.	XU *et al.*, 1981
	A_a-toxin III	22	>9		10.0		Yes	EDTA	XU *et al.*, 1981
Agkistrodon bilineatus									
	Bilitoxin	48		+					IMAI*et al.*, 1989
A. contortrix laticinctus									
	ACL HT-1	29	acidic	DMC, Fg	8.5	α > β		EDTA	JOHNSON and OWNBY, 1993
A. halys blomhoffii									
	HR-I	85	4.7	azocasein, azoalbumin	0.0012		12%	EDTA, cys.	OSHIMA *et al.*, 1972; NIKAI *et al.*, 1986
	HR-II	95	4.2	casein	0.19		7-8%	EDTA, thiols	OSHIMA *et al.*, 1968; OSHIMA *et al.*, 1971
Atractaspis engaddensis		50	acidic		0.2	Yes		EDTA	OVADIA, 1987
Bitis arietans									
	BHRa	68		casein, HPA,gelatin, collagen, insulin B	0.02			EDTA, cys.	OMORI-SATOH *et al.*, 1995; YAMAKAWA *et al.*, 1995
	BHRb	75		casein, HPA,gelatin, collagen, insulin B	0.014			EDTA, cys.	OMORI-SATOH *et al.*, 1995; YAMAKAWA *et al.*, 1995
	HT-1	N. D.	?	casein					MEBS and PANHOLZER, 1982

Table 1 cont'd.

Venom	Toxin	MW (Kd)	pI	Substrates	MHD (µg)	Effect on Fg	+CHO	Inhibitors	Ref.
Bitis gabonica		70							BENNET and MARSH, 1993
Bothrops asper									
	BH1 (BaH1)	64	4.5		0.2			EDTA	BORKOW *et al.*, 1995
	BH2	26	5.2		2.0			EDTA	BORKOW *et al.*, 1993
	BH3	55	5.0		1.6			EDTA	BORKOW *et al.*, 1993
	BaP1	24		casein, HPA, Fg	20	$\alpha > \beta$		EDTA	GUTIÉRREZ *et al.*, 1995
Bothrops jararaca									
	HF1			+	0.1				ASSAKURA*et al.*, 1986
	HF2	50		casein, insulin B	0.02				ASSAKURA*et al.*, 1986; MANDELBAUM *et al.*, 1976
	HF3	62		+	0.015				ASSAKURA*et al.*, 1986
	Bothropasin	48	N. D.	casein, insulin B	N. D.		N. D.	EDTA, EGTA	MANDELBAUM *et al.*, 1982
	Jararhagin	52		fibrin					PAINE *et al.*, 1992
Bothrops moojeni									
	Protease A	20.4	7.7	casein	weak			EDTA	ASSAKURA *et al.*, 1985
Bothrops neuwiedi									
	NHF_a	46	4.2	casein				EDTA	MANDELBAUM *et al.*, 1984
	NHF_b	58	4.3	casein				EDTA	MANDELBAUM *et al.*, 1984
Calloselasma rhodostoma									
	Rhodostoxin	23.4				Yes	15 %		CHUNG *et al.*, 1993, 1996
	HP-1	38							BANDO *et al.*, 1991
Cerastes cerastes									
	Cerastase F-4	22.5			200				DAOUD *et al*, 1986
Crotalus adamanteus									
	Proteinase H	85.7	6.1	casein	0.02			EDTA	KURECKI and KRESS, 1985
Crotalus atrox									
	HT*a*	68	6.1	insulin B	0.04			EDTA	BJARNASON and TU, 1978; OWNBY *et al.*, 1978
	HT*b*	24	basic	Fg	3.0	$\alpha > \beta$		EDTA	BJARNASON and TU, 1978; OWNBY *et al.*, 1978

Table 1 cont'd.

Venom	Toxin	MW (Kd)	pI	Substrates	MHD (μg)	Effect on Fg	+CHO	Inhibitors	Ref.
	HT*c*	24	6.0	casein	8.0			EDTA	BJARNASON and TU, 1978; BJARNASON and FOX, 1987
	HT*d*	24	6.1	casein	11.0			EDTA	BJARNASON and TU, 1978; BJARNASON and FOX, 1987
	HT*e*	25.7	5.6	casein	1.0			EDTA	BJARNASON and TU, 1978
	HT*f*	64	7.7	insulin B, Fg	0.53	γ			NIKAI *et al.*, 1984
	HT*g*	60	6.8		1.4				NIKAI *et al.*, 1985*a*
Crotalus basilicus basilicus									
	B-1	27		+	<10				MOLINA *et al.*, 1990
	B-2	27.5		+	<10				MOLINA *et al.*, 1990
Crotalus horridus horridus									
	Protease IV (HP IV)	57	5.1	Fg, azocoll	4.0	α > β		EDTA	CIVELLO *et al.*, 1983*a, b*
Crotalus ruber ruber									
	HT-1	60	5.8	Fg	0.17	α > β		EDTA	MORI *et al.*, 1987
	HT-2	24	5.2	Fg	0.27	α		EDTA	MORI *et al.*, 1987
	HT-3	25	9.6	Fg	1.43	α		EDTA	MORI *et al.*, 1987
Crotalus scutulatus scutulatus									
	P-13	27		+	1-2				MARTINEZ *et al.*, 1990
Crotalus viridis viridis									
		54		DMC, azocasein, casein, azoalbumin, azocoll, HPA	0.11	α & β	N. D.	EDTA, DTT, phen	KOMORI *et al.*, 1994
		68	8.5	DMC, Fg	N. D.	α	+	EDTA, $NaIO_4$, PNGaseF	LI *et al.*, 1993
		62	4.1	DMC, Fg	N. D.	α	+	EDTA, $NaIO_4$, PNGaseF	LI *et al.*, 1993
Lachesis muta muta									
	LFH-I	100		insulin B, Fg			+		SÁNCHEZ *et al.*, 1987; SÁNCHEZ *et al.*, 1995

Table 1 cont'd.

Venom	Toxin	MW (Kd)	pI	Substrates	MHD (μg)	Effect on Fg	+CHO	Inhibitors	Ref.
	LHF-II	22.3	6.6	insulin B, Fg, casein, DMC, HPA, fibrin	N. D.	α	N. D.	EDTA, EGTA, phen.	SÁNCHEZ *et al.*, 1991
Notechis scutatus scutatus									
	HT_a-HT_i	18-21	acidic						FRANCIS *et al.*, 1993
Ophiophagus hannah									
	Hannahtoxin	66		+	0.7				TAN and SAIFUDDIN, 1990
Philodryas olfersii									
	I	58-70		casein				EDTA, cys.	ASSAKURA *et al.*, 1992
	II	47		Fg, casein		α> β		EDTA, cys.	ASSAKURA *et al.*, 1992
Trimeresurus elegans									
	T. elegans hemorrhgic toxin	28.5	7.4	Fg, DMC, HPA, insulin B	10	β		N-BS, BZA	NIKAI *et al.*, 1991
Trimeresurus flavoviridis									
	HR1a	60	4.4	casein	0.016		17-18%	EDTA, cys.	OMORI-SATOH and SADAHIRO, 1979
	HR1b	60	4.4	casein	0.01		17-18%	EDTA, cys.	OMORI-SATOH and SADAHIRO, 1979; TAKEYA *et al.*, 1990
	HR-2a	23	8.6	Fg	0.07	α		EDTA, cys.	TAKAHASHI and OHSAKA, 1970; NIKAI *et al.*, 1987
	HR-2b	25.5	9.2	Fg	0.07	α		EDTA, cys.	TAKAHASHI and OHSAKA, 1970; NIKAI *et al.*, 1987
Trimeresurus gramineus									
	HR1	23.5	>10	Fg, casein, azocoll	2.5-5.0	α	4%	EDTA	HUANG *et al.*, 1984
	HR2	81.5			1.3-2.5	α, β	10%	EDTA?	HUANG *et al.*, 1984
	β-fibrinogenase	25	4.5	Fg		β > α	7%	PMSF	OUYANG and HUANG, 1979

Table 1 cont'd.

Venom	Toxin	MW (Kd)	pI	Substrates	MHD (µg)	Effect on Fg	+CHO	Inhibitors	Ref.
Trimeresurus mucrosquamatus									
	Mucrotoxin a	94	4.3	casein	2.31			EDTA, DFP	SUGIHARA *et al.*, 1983
	HR-a	15	4.7	Fg, insulin B	1.7	β		EDTA, phen.	NIKAI *et al.*, 1985*b*
	HR-b	27	8.9	Fg, insulin B	2.3	α			NIKAI *et al.*, 1985*b*
Vipera ammodytes ammodytes									
	HT-1	60	basic	insulin B					BJARNASON and FOX, 1994
	HT-2	60	acidic	insulin B					BJARNASON and FOX, 1994
	HT-3	60	acidic	insulin B					BJARNASON and FOX, 1994
Vipera aspis aspis									
		67			0.11				KOMORI and SUGIHARA, 1988
Vipera berus berus									
	HMP	56.3	N. D.	casein, Fg, insulin B	N. D.	α	N. D.	EDTA	SAMEL and SIIGUR, 1990
Vipera lebetina									
	Lebetase	23.7		+					SIIGUR and SIIGUR, 1991
Vipera palestinae									
	HR1	60	basic	gelatin, casein	0.2		+		OVADIA, 1978
	HR2	60	weak acidic	-on casein, gelatin	0.2				OVADIA, 1978
	HR3	60	acidic	- on casein, gelatin	0.4				OVADIA, 1978

Abbreviations: MHD: minimum hemorrhagic dose (µg/g); cys.: cysteine; DMC: N,N-dimethylated casein; Fg: fibrinogen; phen: 1,10-phenanthroline; BZA: benzamidine-HCl; N-BS: *N*-bromosuccinimide; HPA: hide powder azure; -: not detected+: property detected, not quantified; N. D.: not determined.

Comparative analyses of amino acid sequences of snake venom toxins have also revealed similarities in the sequences of hemorrhagic toxins and some putative disintegrin precursor molecules (KINI and EVANS, 1992). These similarities have led to the hypothesis that snake venom metalloproteinases, including hemorrhagic toxins, and disintegrins (platelet aggregation inhibitors) may be derived by proteolytic processing of common precursor proteins comprised of four distinct domains, designated A-D. The metalloproteolytic domain is proposed to lie within domain D while domain C is the disintegrin-like domain. Indeed, some hemorrhagic toxins have been found to contain both a metalloproteolytic site and a disintegrin or disintegrin-like domain (TAKEYA *et al.*, 1993*b*; BJARNASON and FOX, 1995 *a*, *b*).

Mechanism of action of snake venom hemorrhagic toxins

The aforementioned zinc-binding active site and its associated proteolytic activity are currently thought to be keys to the mechanism of biological action of snake venom hemorrhagic toxins. As previously mentioned, these toxins cause hemorrhage by disrupting the microvasculature of tissues. There are two possible mechanisms by which hemorrhage may occur: *per rhexis* and *per diapedesis*. In hemorrhage *per rhexis*, the endothelium and often its associated basal lamina is disrupted or destroyed while the intracellular junctions remain intact. In hemorrhage *per diapedesis* the intracellular junctions of endothelial cells are opened, allowing erythrocytes to escape. Ultrastructural studies have shown that in many snake venoms hemorrhagic toxins cause hemorrhage *per rhexis* (OWNBY, 1990). However, some hemorrhagic toxins are thought to cause hemorrhage *per diapedesis* (OHSAKA, 1979; TSUCHIYA *et al.*, 1978).

Until recently very little work had been done on the direct effects of venoms and hemorrhagic toxins on endothelial cells. One recent study

demonstrated that different hemorrhagic toxins exhibited similar cytotoxic effects on endothelial cells in culture as determined by a neutral red dye assay (OBRIG *et al.*, 1993). In that study hemorrhagic toxins from *C. ruber ruber* (HT-1 and HT-2) and *C. atrox* (atroxase, HT-a and HT-d) were found to be cytotoxic in tissue culture to microvascular endothelial cells. They were not cytolytic. *In vivo* cell lysis seems necessary for hemorrhage to occur. Another study was conducted using cultured murine microvascular endothelial cells treated with a hemorrhagic metalloproteinase as well as a non-hemorrhagic PLA_2-like myotoxin (LOMONTE *et al.*, 1994). In that study no cytotoxicity was observed in the hemorrhagic toxin-treated cells. These cells, however, were observed to detach from their substratum. The myotoxin was shown to be cytotoxic to these same cells by causing lysis of their plasma membranes. Thus, the conflicting data from these two studies have created some controversy regarding the mechanism of action of snake venom hemorrhagic toxins.

It is likely that there is more than one mechanism of action for the many different known hemorrhagic toxins. In general, hemorrhagic toxins are thought to digest or disrupt the proteins comprising the endothelial basal lamina, leading to subsequent endothelial cell disruption and hemorrhage *per rhexis* (TAKEYA *et al.*, 1993*a*). The recent studies mentioned above on the effects of hemorrhagic toxins on endothelial cells *in vitro* would seem to support this theory (OBRIG *et al.*, 1993; LOMONTE *et al.*, 1994; BORKOW *et al.*, 1995). Some hemorrhagic toxins have also been shown to proteolytically degrade proteins of the capillary basal lamina *in vitro* (BARAMOVA *et al.*, 1989). The *in vitro* degradation of basal lamina proteins, however, occurs over a period of several hours whereas hemorrhage induced *in vivo* by hemorrhagic toxins is known to occur within minutes or even seconds (OWNBY, 1990). Electron micrographs of capillaries, in cases of snake venom/toxin induced hemorrhage, often possess intact or only partially

disrupted basal laminae (OWNBY *et al.*, 1978; OWNBY and GEREN, 1987). *In vitro* assays have shown hemorrhagic toxins to be general proteases exhibiting substrate specificity on a wide variety of different protein substrates. Many of these proteins are unrelated to the basal lamina. The degradation of basal lamina proteins *in vitro* is therefore not conclusively indicative of the mechanism of hemorrhagic toxin action. It cannot be ruled out, however, that this action may play a significant role in the production of hemorrhage *in vivo* . It is possible that degradation of basal lamina proteins and direct cytotoxicity are both involved. At any rate, whether microvascular endothelial cell disruption *in vivo* in hemorrhage *per rhexis* is a primary or secondary event involving snake venom hemorrhagic toxins is unclear.

Other possible mechanisms of hemorrhage include indirect effects of hemorrhagic toxins. The fibrinogenolytic and fibrinolytic effects and coagulopathies induced by snake venom hemorrhagic toxins have been implicated in the induction and complication of hemorrhage (SUGIKI *et al.*, 1995). Many hemorrhagic toxins act on fibrinogen and/or fibrin *in vitro*, a finding that suggests that these toxins may also have an anticoagulant effect exacerbating hemorrhage that may be induced by other mechanisms (TU, 1988; KAMIGUTI *et al.*, 1992; DECLERCK *et al.*, 1994).

As previously mentioned, snake venom hemorrhagic toxins may act upon several protein substrates associated with microvascular endothelial cells to elicit hemorrhage. The substrates include the aforementioned basal lamina proteins and possibly other proteins expressed/secreted by endothelial cells. Two particularly intriguing possibilities are von Willebrand Factor (vWF), also known as Factor VIII-related antigen (Factor VIII:RAg), and a distinctly different clotting factor, Factor VIII:C. vWF is necessary for the activation of Factor X which is responsible for the conversion of prothrombin to thrombin (KORNALIK,

1991). A systemic deficiency in Factor VIII:C results in classical type A hemophilia (BANERJEE *et al.*, 1992). Deficiencies in vWF and Factor VIII can result in severe hemophilia. Generally, severe local hemorrhage is commonly associated with hemophilia. It is possible that hemorrhagic toxins could induce hemorrhage be creating local and sometimes even systemic deficiencies or irregularities in these clotting factors.

The effects of hemorrhagic toxins on endothelial cells and endothelial cell responses to injury

Endothelial cells, particularly those forming capillaries (i. e. microvascular endothelial cells), have been shown by light and electron microscopy to undergo degeneration and necrosis from *in vivo* injection of various snake venom hemorrhagic toxins (OWNBY *et al.*, 1978; MOREIRA *et al.*, 1994). Currently the only evidence of direct damage to vascular endothelial cells *in vitro* does not correlate well with the extent and timing of damage seen *in vivo*. *In vivo* hemorrhage *per rhexis* occurs very rapidly, in most cases within 5-10 minutes after injection. Toxic effects of hemorrhagic toxins on cultured endothelial cells (OBRIG *et al.*, 1993) and the proteolysis of substrates by hemorrhagic toxins *in vitro* (BARAMOVA *et al.*, 1989) require much longer periods of time to occur. The discrepancies between the *in vivo* and *in vitro* data could be explained by simple difference in the experimental conditions and in phenotypic expressions of these cells in the different environments. Injury to endothelial cells is characterized by release of specific markers such as vWF, thrombomodulin, and angiotensin converting enzyme. Release of cytokines and vasoactive substances, changes in adhesion molecule expression, complement activation, and morphological changes are also indicative of endothelial cell injury (Fig. 1) (GRACE, 1994). These responses lead to a series of events

including vasoconstriction, alterations in platelet aggregation, and neutrophil (PMN) chemotaxis and PMN adherence which can often exacerbate hemorrhage. Initial damage to the endothelium such as is seen with hemorrhagic toxins undoubtedly leads to further complications brought on by these responses. However, in their examination of thirty-eight clinical cases of envenomation by *Bothrops jararaca*, a notoriously hemorrhagic venom, KAMIGUTI *et al.* (1992) did not detect elevated levels of endothelial cell injury markers (e. g. thrombomodulin and von Willebrand factor), indicating that there was no evidence of direct damage to endothelial cells *in vivo*.

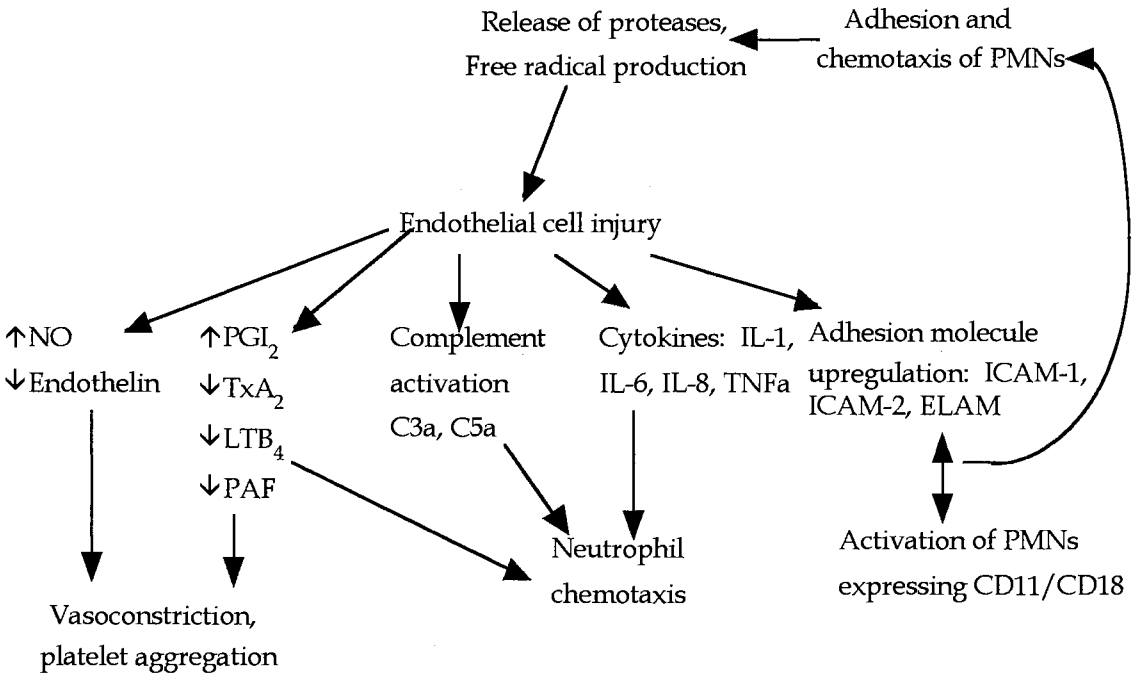

FIG. 1 RESPONSES OF ENDOTHELIAL CELLS TO INJURY AND THEIR CONSEQUENCES

Aims of the study

In 1799, the Eastern diamondback rattlesnake (*Crotalus adamanteus*) was one of the first venomous snakes described as a North American species (COLLINS, 1990). Since that time it has been a snake of considerable medical importance as it possesses one of the most hemorrhagic snake venoms found in North America (HOMMA and TU, 1971). Incidentally, this venom is also one of the four venoms used in the production of the Wyeth polyvalent (Crotalidae) antivenom and as such it seems a suitable subject for a study on the elucidation of the effects of a hemorrhagic toxin on endothelial cells.

A better understanding of the mechanism of hemorrhagic toxins at the cellular level is needed if a suitable means of preventing or diminishing the effects of hemorrhage is to be found. The work that follows is a study of one important snake venom hemorrhagic toxin, proteinase H from *Crotalus adamanteus* venom. Three hypotheses are tested in this dissertation. The first hypothesis is that the hemorrhagic and proteolytic activity of proteinase H is dependent upon its glycosylation. The second hypothesis is that proteinase H induces hemorrhage *per rhexis*. The third hypothesis is that proteinase H is directly cytotoxic to human dermal microvascular endothelial cells in culture. In addition to testing these hypotheses, several of the biochemical and physical characteristics of proteinase H are reported. The pathogenesis of local and systemic hemorrhage induced by proteinase H in mice is described and the effects of proteinase H on different types of cultured endothelial cells are reported.

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CHAPTER II

FIBRINOGEN-CLOTTING ACTIVITY OF PROTEINASE H, THE HEMORRHAGIC ZINC-METALLOPROTEINASE FROM *CROTALUS ADAMANTEUS* (EASTERN DIAMONDBACK RATTLESNAKE) VENOM

ABSTRACT

Proteinase H, the sole hemorrhagic factor in the venom of the Eastern diamondback rattlesnake, was first isolated from crude venom by KURECKI AND KRESS in 1985. In the current study, proteinase H was isolated using a modification of their method. Its fibrinogen-clotting activity was quantified as 5.0 NIH Units/mg of protein. Proteinase H preferentially releases fibrinopeptide B (FPB) and digests the Bβ subunit of fibrinogen. The fibrinogen-clotting activity of proteinase H was not inhibited by treatment with phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, ethylenediaminetetraacetic acid (EDTA), a divalent metal cation chelator, or deglycosylation by treatment with peptide *N*-glycosidase from *Flavobacterium meningosepticum* (PNGaseF). These data suggest that the fibrinogen-clotting activity of proteinase H is not due to serine protease activity, and the activity is not dependent on zinc nor the presence of carbohydrate moieties. To

our knowledge this is the first report on the influence of a snake venom hemorrhagic metalloproteinase on fibrinogen-clotting *in vitro*.

INTRODUCTION

Proteinase H was first isolated by KURECKI AND KRESS in 1985 as the sole hemorrhagic metalloproteinase in *Crotalus adamanteus* venom. It is a zinc-dependent proteolytic glycoprotein with a molecular weight of 85.7 kD and a pI of 6.1. EDTA was found to inhibit its proteolytic/hemorrhagic activity while PMSF had no such inhibitory effect. We have found that this same toxin also possesses a thrombin-like property of causing fibrinogen to clot by the formation of a network of fibrin polymers. This was serendipitously discovered during the routine assay for fibrinogenolytic activity in which instead of degradation of fibrinogen into its respective subunits, as is commonly found with this class of proteins, the formation of an opaque, dissoluble clot occurred. The study that follows describes the purification procedure used in obtaining proteinase H as well as the characterization of its fibrinogen-clotting activity.

MATERIALS AND METHODS

Venom and reagents

Crude *C. adamanteus* (Eastern diamondback rattlesnake) venom from a single pool of snakes from Florida was purchased in lyophilized form from Biotoxins, Inc. (St. Cloud, FL, U.S.A.). 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPS) was purchased from Calbiochem (La Jolla, CA, U.S.A). *N,N*-dimethlyated casein, bovine thrombin, bovine fibrinogen,

TRIZMA® base (Tris[hydroxymethyl] aminomethane), silver nitrate, sodium bicarbonate, 2,4,6-trinitrobenzenesulfonic acid (TNBS), PMSF, Gly-His-Pro-Arg (acetate salt), ammonium acetate, and sodium periodate ($NaIO_4$) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ethylenediamine tetraacetic acid, disodium salt (EDTA) was purchased from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A). Peptide *N*-glycosidase F from *Vibrio cholerae* (PNGaseF) was purchased from Boerhinger Mannheim Corp. (Indianapolis, IN, U. S. A.). PhastGel electrophoresis media and PhastGel Blue R staining kit for Coomassie blue visualization were purchased from Pharmacia (Piscataway, NJ, U. S. A.). Acetonitrile was purchased from Aldrich Chemical Co. (Milwaukee, WI, U. S. A.).

Purification of proteinase H

Proteinase H was isolated by a modification of the liquid chromatographic series reported by KURECKI AND KRESS (1985). The procedure was modified by replacing the final, anion exchange open column step with an HPLC column for greater resolution. In each step the volumes specified are collected in test tubes and assayed for absorbance at 280 nm and caseinolytic activity. Tubes were pooled according to caseinolytic activity or, in the absence of such activity, to absorbance at 280 nm. These pools are referred to as fractions. Fractions containing caseinolytic material were then tested for hemorrhagic activity. Hemorrhagic fractions were selected for further purification as described. Crude *C. adamanteus* venom (2.0 g) was dissolved in 0.02 M sodium borate buffer (pH 8.0) containing 0.1 M NaCl and 10 % ethylene glycol and initially fractionated on a CM Bio-Gel A cation exchange column (2.6 X 27 mm). The column was eluted at a flow rate of 20 ml/hr using a step-wise

NaCl concentration gradient beginning with 0.1 M NaCl then increasing the concentration to 0.3 M and finally to 1.0 M. Five milliliter volumes were collected and assayed for caseinolytic activity. Fraction A1 was concentrated and dialyzed against 0.05 M Tris-HCl (pH 8.0). After dialysis solid KCl was added to a final concentration of 1 M. Fraction A1 was then fractionated by hydrophobic interaction chromatography on a Phenyl Sepharose column (2.6 X 25 cm). The column was eluted at a flow rate of 50 ml/hr using a step-wise gradient from the starting buffer to 0.05 M Tris-HCl (pH 8.0) and finally to 0.05 M Tris-HCl (pH 8.0) with 50 % ethylene glycol. Five ml volumes were collected and assayed for hemorrhagic and caseinolytic activity. Fraction B3 was concentrated and dialyzed against 0.1 M Tris, 0.5 M NaCl (pH 8.6), then separated by gel filtration chromatography on a Sephacryl S-200 HR column (2.6 X 100 cm) eluted with the aforementioned buffer at a flow rate of 20 ml/hr. Five ml volumes were collected and assayed for hemorrhagic and caseinolytic activity. Fraction C1 was concentrated and dialyzed against 0.05 M Tris (pH 8.0). This material was then separated by semi-preparative anion exchange HPLC on a Waters DEAE-5PW column. The column was eluted at 3 ml/min using a linear NaCl concentration gradient. Peaks were detected by absorbance at 280 nm and 1 ml volumes were collected and assayed for hemorrhagic activity. Fraction D2 was determined to be homogeneous and is hereafter referred to as proteinase H. Homogeneity was demonstrated by the formation of a single band on an SDS-PAGE gel stained with silver nitrate and by obtaining a single peak on analytical C18 reversed-phase HPLC.

Determination of hemorrhagic activity

The hemorrhagic activity of fractions was determined by intradermal injection of 100 µl samples into the backs of CD-1 white female mice (Charles River). After three hrs the mice were killed and the skin from the backs of the treated mice removed. If present, a spot of hemorrhage was quantified by measuring the widest diameter of the hemorrhagic spot and the diameter of the spot at a right angle to the first measurement (OWNBY *et al.*, 1984). These data were expressed as hemorrhagic indices that were calculated by multiplying these measurements and were expressed in mm^2.

Deglycosylation of proteinase H with PNGaseF

Proteinase H was deglycosylated as described in LI *et al.* (1993). Briefly, 0.4 U of PNGaseF in 2 µl was mixed with 10 µl of a 1 mg/ml solution of proteinase H and the volume made up to 50 µl with 25 mM Tris buffer (pH 7.3). This mixture was incubated overnight at 37°C. Deglycosylation was confirmed by a negative reaction with PAS stain on an SDS-PAGE gel.

Test-tube clotting assay

To quantify the fibrinogen-clotting activity of the toxin the test-tube clotting assay described by DOWD *et al.* (1995) was used. Fifty microliters of a solution containing the proteinase H sample to be tested was added to 100 µl of 30 mg/ml bovine fibrinogen in phosphate buffered saline (pH 7.4) in microcentrifuge tubes. The final concentration of fibrinogen was 20 mg/ml. The tubes were incubated at 37°C and the time taken for fibrin clots to form was recorded. If clots did not form within 12 hrs. the sample was considered inactive.

A thrombin standard curve was generated using the same method to estimate the clotting activity of proteinase H on fibrinogen. Thrombin was added to the fibrinogen solution as described above in the following amounts: 5, 3, 1, 0.5, 0.3, 0.1, 0.05 and 0.03 NIH Units. The specific fibrinogen-clotting activity of the proteinase H treatments described above were estimated by comparison to this thrombin standard curve and expressed in NIH Units/mg.

Fibrinogen-agarose plate assay

The fibrinogen-agarose plate assay of DOWD *et al.* (1995) was used. Fibrinogen-agarose plates were made by mixing 10 ml of 1% agarose warmed to 50°C with 9.5 ml of 0.4% (w/v) fibrinogen in PBS. This mixture was poured into a petri dish (10 cm diam.) and allowed to cool to room temperature to solidify. Wells (4 mm diameter) were cut in the agarose and 5 µl aliquots of thrombin or proteinase H added to the wells. The plates were covered and then incubated for 5 hours at 37°C in a humidified chamber. Following incubation the diameters of rings formed around the wells due to fibrin polymerization were measured.

Electrophoretic analysis of fibrin clots

Fibrin clots produced in 1 ml microfuge tubes during the test-tube clotting assay were subjected to reducing SDS-PAGE analysis as described by DOWD *et al.* (1995). Fibrin clots were formed in microfuge tubes and then centrifuged at 15,000 X *g* for 15 minutes. The supernatant was removed and the pelleted clot washed five times by the addition of 1 ml of PBS, followed by centrifugation at 15,000 X *g* for 15 minutes. After the final wash the pellet was resuspended in 100 µl of SDS-PAGE sample buffer and heated to 100°C for 5 minutes. Reducing SDS-PAGE was performed on 12.5 % homogeneous gels using the Pharmacia

PhastSystem electrophoresis unit. Gels were visualized by staining with Coomassie blue.

Effect of inhibitors on clotting activity of proteinase H

The effect of various potential inhibitors on the fibrinogen-clotting activity of proteinase H was determined by the test tube clotting assay described above following treatment of the toxin with each inhibitor. To determine the effect of inhibitors on the fibrinogen-clotting time of proteinase H a 1 mg/ml aqueous solution of proteinase H was mixed with an equal volume of either 50 mM EDTA (pH 7.2), 8 mM of PMSF in DMSO, or 150 μM Gly-His-Pro-Arg (a fibrin anti-polymerant) prior to the experiment. Following incubation PMSF was removed by dialysis. Proteinase H was deglycosylated using the *N*-glycosidase PNGaseF by incubating a solution of 50 μg of proteinase H with 0.4 U of PNGaseF overnight at 37°C. The thrombin-clotting time of each preparation and electrophoretic analysis of the resultant clots were conducted as described above. Electrophoretic analysis was not conducted on clots formed by anti-polymerant-treated proteinase H.

Defibrinating activity

Proteinase H in 10 mM Tris-buffered saline (pH 7.5) was injected at doses of 0.5 μg/g and 1.0 μg/g intravenously via the ventral median tail vein into female white mice (CD-1, Charles River) weighing 22-24 grams. After one hour the mice were bled from the infraorbital sinus, using 20 μl non-heparinized glass microcapillary tubes. The tubes were allowed to sit at room temperature and checked for clotting every ten minutes for 1 hr by sequentially breaking the tube from the top down. Lack of clotted blood in tubes was considered positive for defibrinating activity.

Fibrinolytic activity

To assay for fibrinolytic activity the fibrin plate assay described by CHEN *et al.* (1991) was used. Proteinase H was applied to fibrin gel plates and incubated for 4 hours at 37°C in a humidified chamber. Fibrinolytic activity was indicated by the appearance of a zone of clearing in the plate surrounding the spot of sample application.

Reversed-phase HPLC analysis of fibrinopeptides

Release of fibrinopeptides A (FPA) and B (FPB) were analyzed as described by MOSESSON *et al.* (1987). Bovine fibrinogen was brought to a concentration of 5 mg/ml in 150 mM NaCl, 50 mM Tris (pH 7.2), and incubated in microfuge tubes after the addition of either 0.01 U thrombin or 10 µg of proteinase H. Release of FPA and/or FPB in 100-300 µl of supernatant solution from each tube was analyzed by RP-HPLC on a µBondapak C18 column (3.9 X 300 mm) eluted with a linear gradient of acetonitrile (12% to 28%) buffered with 25 mM ammonium acetate (pH 6.0). The acetonitrile gradient was performed over 45 minutes with a flow rate of 1 ml/min. Peptides were detected by absorbance at 210 nm.

Transmission electron microscopic analysis of fibrin clots

Fibrin clots were formed on 200 mesh carbon-coated, Formvar copper grids as described in WEISEL (1986). The grids were first dipped in a solution containing 10 NIH U/ml of either thrombin or proteinase H. After coating with these clotting agents, 25 µl drops of fibrinogen (2 mg/ml in 150 mM NaCl, 50 mM Tris, pH 7.2) were applied to each grid. These grids were then incubated overnight at room temperature (approximately 20°C) in a humidified container. Following incubation the grids were removed from the now clotted fibrinogen

droplets and negatively contrasted with 2% uranyl acetate in 10% acetone for 30 seconds. The fibers thus prepared were then examined by TEM at 80 kV at 4,800, 10,000, and 100,000 times magnification.

RESULTS

Purification of proteinase H

Cation exchange chromatography of crude venom on CM Bio-Gel A yielded a hemorrhagic fraction containing 812.6 mg of material (Fig 1A). Tubes 20-42 containing hemorrhagic material were pooled as fraction A1. Further purification of this hemorrhagic fraction was carried out by hydrophobic interaction chromatography (HIC) on Phenyl-Sepharose column. Tubes 170-177 contained all detectable hemorrhagic activity and were pooled as fraction B3. Fraction B3 (approximately 35 mg) was the only hemorrhagic fraction to elute from the column. It was eluted by 0.05 M Tris-HCl, pH 8.0 (Fig. 1B). This material was concentrated, dialyzed against 0.1 M Tris (pH 8.6) containing 0.5 M NaCl, and subjected to gel filtration chromatography on a Sephacryl S-200 HR column (Fig. 1C). Tubes 40-65 contained all detectable hemorrhagic activity and were pooled as fraction C1. The high molecular weight fraction (C1) was the only hemorrhagic fraction eluting from the column. A total of 20.1 mg of material was collected in this fraction and purified further by DEAE anion exchange HPLC (Fig. 1D). Tubes 50-56 contained all detectable hemorrhagic activity and were pooled as fraction D2. Fraction D2 began eluting from the DEAE column at a NaCl concentration of approximately 0.23 M. A total of 13.1 mg of protein was obtained (Table 1). This material was judged to be homogeneous by virtue of the formation of a single band on SDS-PAGE stained

with silver nitrate (Fig. 2) and by analytical C18 RP-HPLC (Fig. 3) and was used for these studies.

Fibrin clot formation by proteinase H

When 10 μg of proteinase H was added to a solution of bovine fibrinogen a stable, gel-like clot formed within 152 ± 22 minutes (n=3). The clot thus formed was distinguishable from that formed by thrombin in that the proteinase H-generated clot was more opaque. This increased opacity was evident in the fibrinogen-agarose plate assay as a distinct, opaque ring (Fig. 4). Pre-treatment of the enzyme with EDTA, PMSF, or PNGaseF did not inhibit this activity (Table 2). Thus, proteinase H had a clotting activity equal to 5.0 X 10^{-3} NIH Units of thrombin, giving proteinase H an estimated specific clotting activity of 5.0 NIH Units/mg protein. The thrombin standard to which proteinase H was compared had a specific activity of 105 NIH U/mg.

Defibrinating and fibrinolytic activity

Proteinase H exhibited no defibrinating action when injected intravenously into mice. Clot formation was observed in as little as ten minutes. Proteinase H did not create a zone of clearing in a fibrin gel plate, indicating the absence of any fibrinolytic activity.

Effect of inhibitors and deglycosylation on fibrinogen-clotting activity

Treatment of proteinase H with 4 mM PMSF or with 25 mM EDTA had no statistically significant effect ($p<0.05$) on its mean thrombin-clotting time (Table 2). This is an interesting note because in other studies by our lab it was found that treatment of proteinase H with either EDTA or PNGaseF abolished its hemorrhagic activity and proteolytic action on Azocoll™, a modified form of

collagen, and *N,N*-dimethlyated casein (Chapter III). However, while EDTA-treatment and deglycosylation did not inhibit clot formation, both inhibited the digestion of the Bβ subunit of fibrinogen (Fig. 5). EDTA-treatment and deglycosylation resulted in the formation of a clot which was physically indistinguishable from that formed by thrombin. Neither EDTA-treatment nor deglycosylation had any effect on the diameter of the precipitin ring formed in the fibrinogen-agarose plate assay (Table 3).

Fibrinopeptide release

Fibrinopeptide release during treatment with proteinase H was compared to that due to treatment with thrombin. The mean peak areas of FPA and FPB released from fibrinogen treated with thrombin were $4.72 \times 10^5 \pm 3.15 \times 10^5$ μV•sec and $1.34 \times 10^6 \pm 1.84 \times 10^5$ μV•sec respectively. In comparison, the mean peak areas of FPA and FPB released from proteinase H-treated fibrinogen were $5.46 \times 10^4 \pm 1.05 \times 10^4$ μV•sec and $1.10 \times 10^6 \pm 1.45 \times 10^5$ μV•sec respectively. Treatment of bovine fibrinogen with proteinase H therefore resulted in the preferential release of FPB. Approximately 82 ± 11 % of the FPB released by treatment with thrombin was released by proteinase H treatment. Conversely, only 12 ± 2 % of the FPA released by thrombin was released by proteinase H.

Electron microscopic analysis of fibrin clot

The fibrin network formed by treatment of fibrinogen with proteinase H was qualitatively similar in appearance to that formed by treatment with thrombin at all the magnifications viewed. This procedure was carried out to confirm that the phenomenon being observed following treatment was due

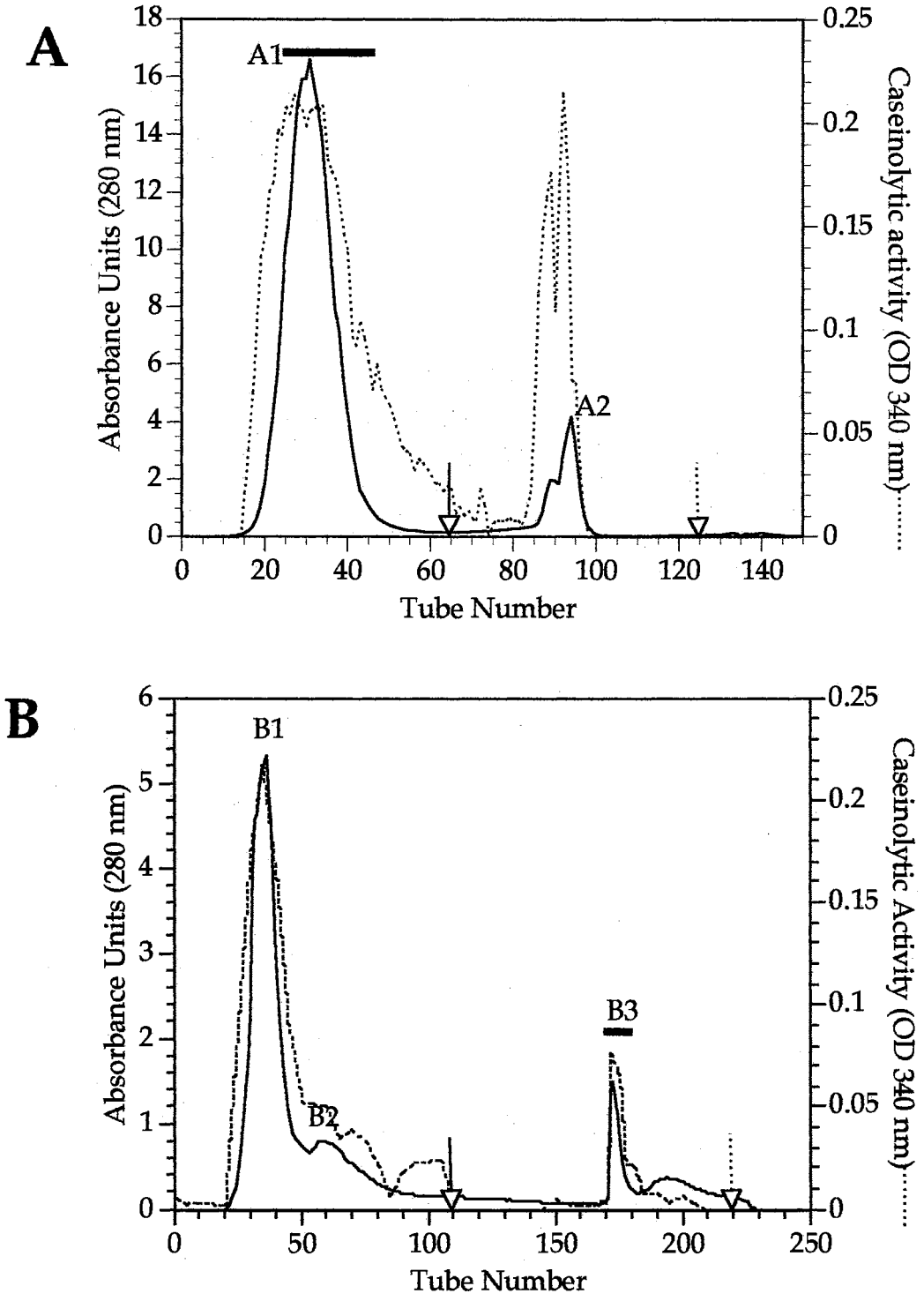
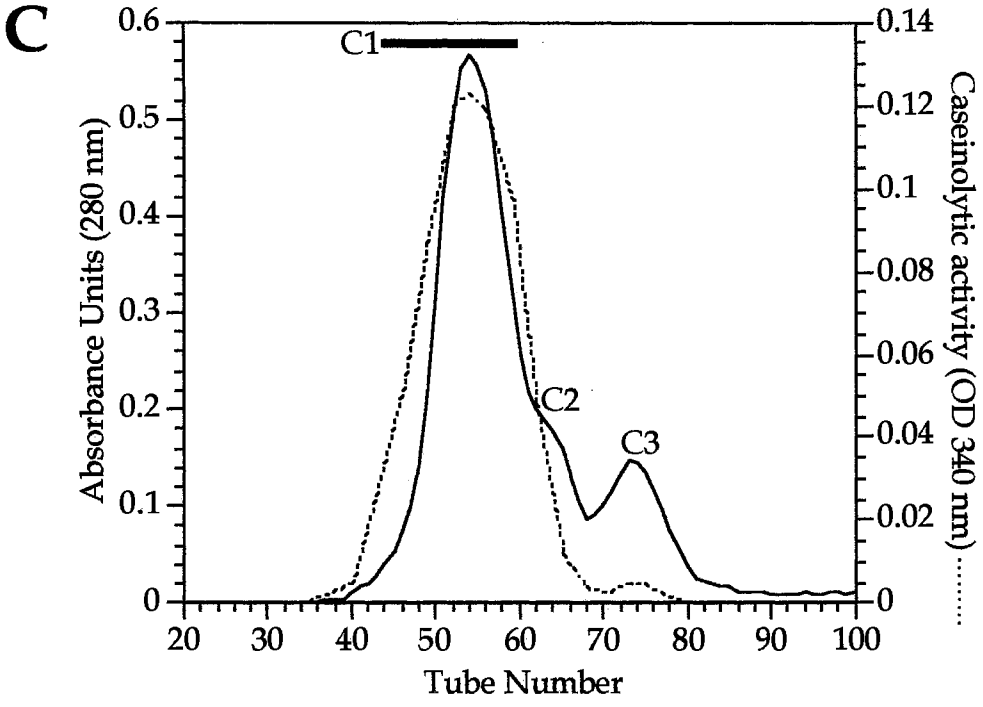

A
A1
A2
Absorbance Units (280 nm)
Caseinolytic activity (OD 340 nm)
Tube Number
B
B1
B2
B3
Absorbance Units (280 nm)
Caseinolytic Activity (OD 340 nm)
Tube Number

FIG. 1 (cont'd). ISOLATION OF PROTEINASE H. (C) Fraction B3 was separated on a Sephacryl S-200 HR column. The column was eluted with 0.1 M Tris, 0.5 M NaCl, pH 8.6 using a flow rate of 20 ml/hr and fractions were collected in 5 ml volumes. (D) Fraction C1 was separated on a Waters DEAE-5PW semi-preparative HPLC column. The column was eluted using a linear NaCl concentration gradient at a flow rate of 3 ml/min. Fractions were collected in 1 ml volumes Fraction D2 was determined to be homogeneous by SDS-PAGE and analytical C18 RP-HPLC.


C
C1
C2
C3
Absorbance Units (280 nm)
Caseinolytic activity (OD 340 nm)
Tube Number
0.6
0.5
0.4
0.3
0.2
0.1
0
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
20
30
40
50
60
70
80
90
100

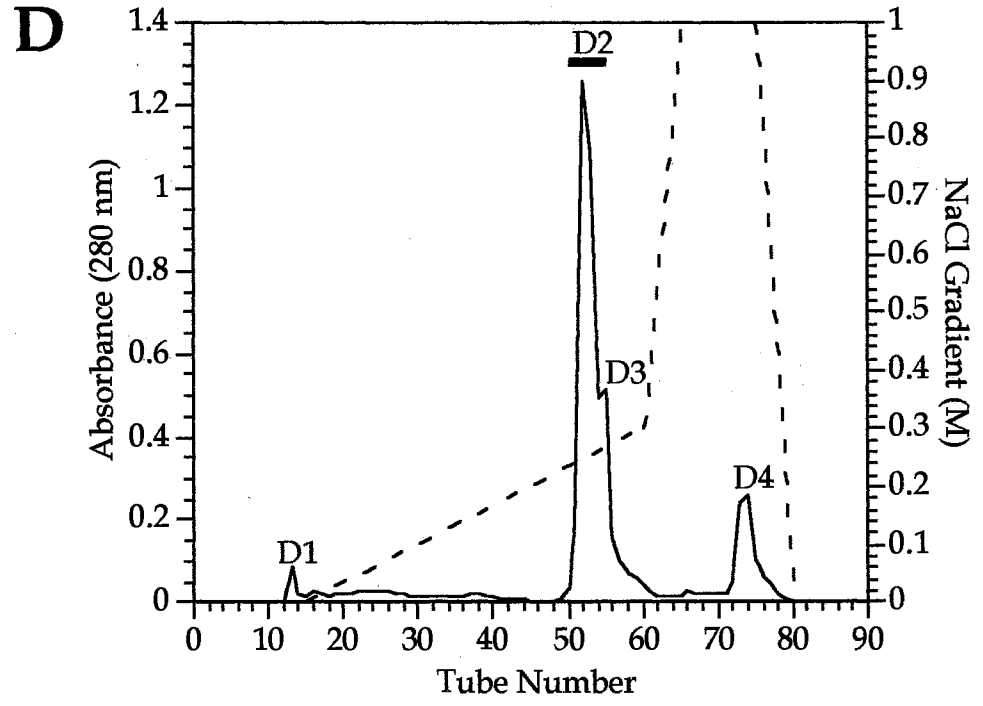

D
D1
D2
D3
D4
Absorbance (280 nm)
NaCl Gradient (M)
Tube Number
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
80
90

FIG. 2 SDS-PAGE OF PROTEINASE H. Native and deglycosylated proteinase H were separated on a 20%T denaturing SDS-PAGE gel and visualized with silver nitrate to demonstrate homogeneity. MW - molecular weight markers; Lane 1 - proteinase H; Lane 2 - deglycosylated proteinase H. Homogeneity is indicated by the formation of a single band.

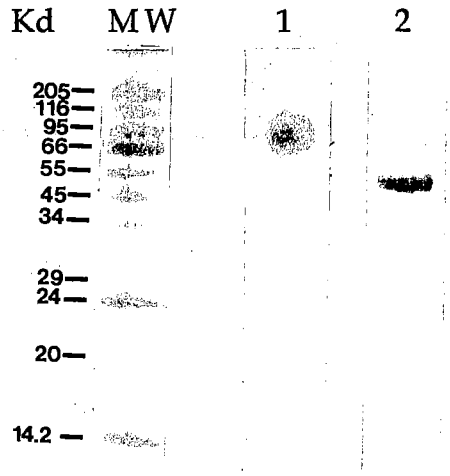

Kd
MW
1
2
205
116
95
66
55
45
34
29
24
20
14.2

FIG. 3 ANALYTICAL C18 RP-HPLC OF PROTEINASE H. Samples of proteinase H was subjected to analytical reversed-phase HPLC on a C18 column eluted with a linear acetonitrile gradient to further verify the homogeneity of the material used in this study. Homogeneity is indicated by the elution of a single peak.

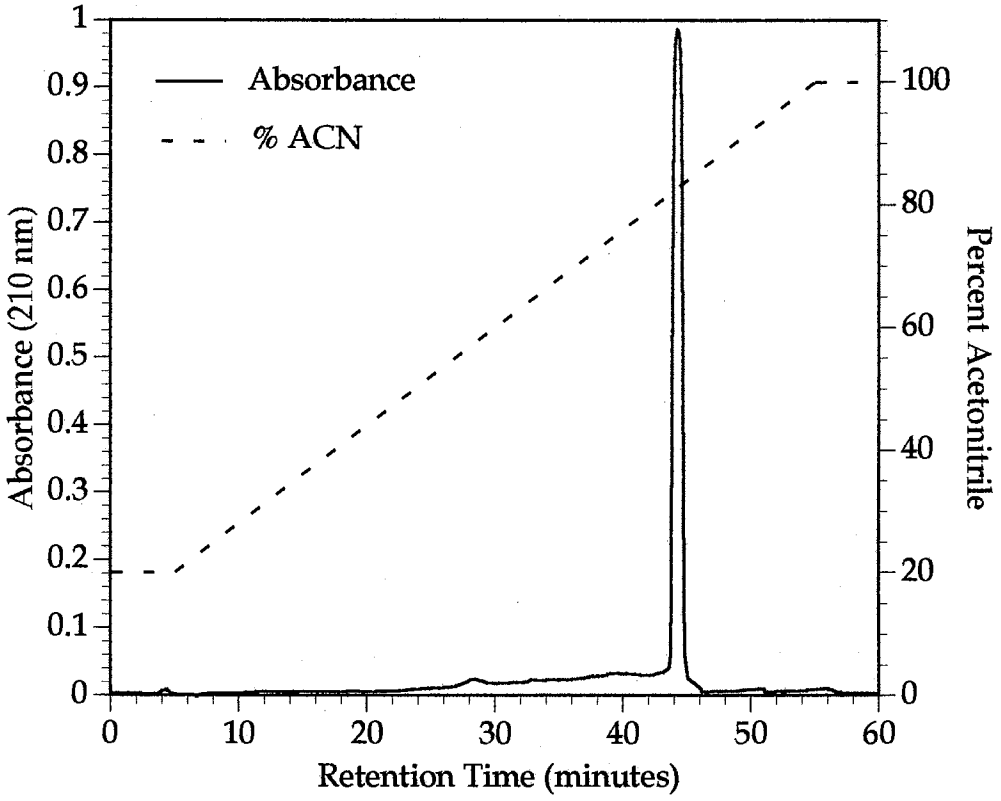

Absorbance
% ACN
Absorbance (210 nm)
Percent Acetonitrile
Retention Time (minutes)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
20
40
60
80
100
0
10
20
30
40
50
60

TABLE 1. SUMMARY OF PURIFICATION OF PROTEINASE H

Step	Yield (mg)	Total Activity (pmoles/min)	Specific Activity‡	MHD (μg/g)	% Yield†	Purification
Crude Venom	2000	176.0	58.5	1.0	100	0
CM Bio-Gel A, Fraction A1	812.6	73.7	90.7	N. D.	63	1.5
Phenyl-Sepharose, Fraction B3	35.0	9.9	282.9	N. D.	8.5	4.8
Sephacryl S-200 HR, Fraction C1	20.1	6.6	328.4	N. D.	5.6	5.6
DEAE-5PW, Fraction D2	13.1	5.6	427.5	0.02	4.8	7.3

‡pmoles/min/mg
*% of total protein yield = mg of fraction/2000 mg
†% of total proteolytic activity = total activity/176 pmoles/min

FIG. 4. SDS-PAGE OF FIBRIN CLOTS. Fibrin clots were formed by treatment with thrombin or with one of the following proteinase H preparations: native proteinase H, PMSF-treated proteinase H, EDTA-treated proteinase H, deglycosylated proteinase H. The clots were solubilized in SDS-PAGE buffer as described in methods and separated on a 12.5 % T acrylamide gel. Lane 1, fibrinogen control; Lane 2, thrombin-generated clot; Lane 3, proteinase H-generated clot; Lane 4, PMSF-treated proteinase H-generated clot; Lane 5, EDTA-treated proteinase H-generated clot; Lane 6 deglycosylated proteinase H-generated clot. The gel was visualized with Coomassie blue.

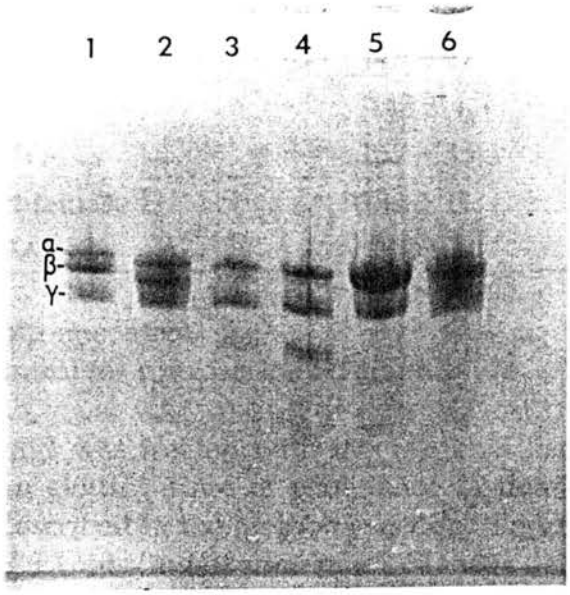

1
2
3
4
5
6
α-
β-
γ-

TABLE 2. EFFECT OF VARIOUS REAGENTS ON THE FIBRINOGEN-CLOTTING ACTIVITY OF PROTEINASE H

Treatment	Mean Clotting Time (minutes)	Specific Activity (NIH U/mg)
[a]Thrombin (0.05 NIH U)	120 ± 36	110
Proteinase H (50 μg)	56 ± 4	2.0
[b]Proteinase H (10 μg)	148 ± 22	5.0
Proteinase H + PMSF	148 ± 4	5.0
Proteinase H + EDTA	152 ± 35	4.2
Deglycosylated proteinase H	133 ± 11	4.5
Proteinase H + GPAP	148 ± 4	3.5

[a]- Thrombin as clotting control.
[b]- Proteinase H control for comparison with inhibitors.

Proteinase H (10 μg) was treated with the reagents listed in the table and the effect on the mean clotting time of proteinase H determined by the test-tube clotting assay as described in the methods section. Specific activity of proteinase H expressed as NIH Units/mg of protein ± 1 SD was determined by comparison of clotting time with those of a thrombin standard curve.

FIG. 5. FIBRINOGEN-AGAROSE PLATE ASSAY. The zone of clotting induced by (A) thrombin or (B) proteinase H in a fibrinogen-agarose plate is shown. The distinct opaque ring (arrowhead) formed by treatment with proteinase H is shown as an example of the physical difference between clots formed by proteinase H and those formed by thrombin.

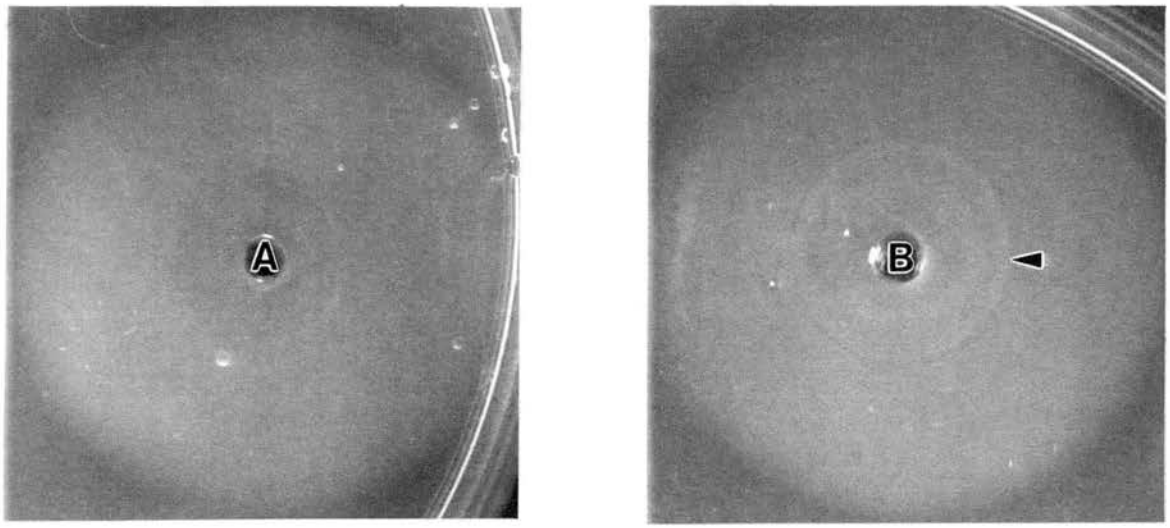

A
B

TABLE 3. DIAMETERS OF PRECIPITIN RINGS FORMED IN FIBRINOGEN-AGAROSE PLATE ASSAY

Treatment	Mean ring diameter (mm)
Thrombin (0.5 U)	18.3 ± 0.6
ProH (10 μg)	13.3 ± 0.6
ProH + 25 mM EDTA	11 ± 1.0
ProH + 4 mM PMSF	12 ± 1.0

Proteinase H was determined to have a fibrinogen-clotting activity of approx. 5 NIH U/mg in an earlier assay. In this fibrinogen-agarose plate assay 0.5 U of proteinase H (10 μg) was used to determine the effects of inhibitors on the fibrinogen-clotting activity of proteinase H. Thrombin (0.5 U) was used as a comparative control.

to the formation of a fibrin network as opposed to simple fibrinogen aggregation or the formation of fibrinogenin as is induced by treatment of fibrinogen with factor XIII alone (BLOMBÄCK, *et al.*, 1990).

DISCUSSION

To date at least sixteen fibrinogen-clotting enzymes have been isolated and characterized from snake venoms (PIRKLE AND THEODOR, 1988, 1990; STOCKER AND MEIER, 1988). The primary activity of these enzymes is the clotting of fibrinogen by the formation of a fibrin network. However, some of these enzymes induce platelet aggregation (NISHIDA *et al.*, 1994), factor XIII activation, cleavage of synthetic esters, activation of prothrombin, protein C (STOCKER AND MEIER, 1988). None of the snake venom fibrinogen-clotting enzymes known has all of these activities. We have shown that proteinase H from *C. adamanteus* venom is a fibrinogen-clotting enzyme. The most interesting aspect of this activity in proteinase H is that it seems contrary to the apparent dominant activity of the toxin, which is to induce hemorrhage. The formation of a fibrin clot in or around an area of hemorrhage would seem at first to oppose the hemorrhagic effect of the toxin. However, the *in vitro* fibrinogen-clotting activity of proteinase H is relatively weak and its hemorrhagic action is potent. The toxin has a MHD of 0.02 μg/g in mice. There is no evidence that proteinase H causes fibrinogen-clotting *in vivo*. Thus it seems unlikely that the fibrinogen-clotting activity of proteinase H plays a role in the pathogenesis of hemorrhage induced by the toxin *in vivo*. What is more likely is that this activity of proteinase H may have a deleterious effect on hemostatic mechanisms *in vivo* at later time periods, perhaps several hours after the initial introduction of the toxin into the body. Continual unregulated activity of such an exogenous

fibrinogen-clotting enzyme could conceivably deplete plasma fibrinogen levels, predisposing the subject to disseminated intravascular coagulation (MCKAY *et al.*, 1970; MAGALHÉS *et al.*, 1981; KORNALIK, 1991). Intravenous injection of one fibrinogen-clotting enzyme from the venom of the bushmaster (*Lachesis muta*) resulted in a 15 % reduction of the total plasma fibrinogen concentration in as little as 30 minutes in dogs (MAGALHÉS *et al.*, 1981). Additionally, the generation of fibrinopeptides may stimulate plasmin activity, exacerbating the problem (KORNALIK, 1991).

In the present study proteinase H was treated with several different agents that were seen as potential inhibitors of its fibrinogen-clotting activity. Specifically, PNGaseF, EDTA and PMSF were tested for any inhibitory effect on the fibrinogen-clotting activity of proteinase H. PNGaseF and EDTA were selected because they have both been shown to have inhibitory effects on the proteolytic and hemorrhagic activities of proteinase H (Chapter III). PMSF was selected as it is a serine protease inhibitor, and the activity of other fibrinogen-clotting or thrombin-like enzymes has been shown to be due to the serine protease activity of those enzymes. As expected, PMSF did not inhibit the fibrinogen-clotting activity of proteinase H nor the digestion of the Bβ subunit of fibrinogen. Treatment of proteinase H with EDTA or PNGaseF, however, also failed to inhibit its fibrinogen-clotting activity. Treatment with EDTA and PNGaseF did inhibit the digestion of the Bβ subunit of fibrinogen, indicating that the major proteolytic activity of proteinase H was inhibited by these two agents. Thus, inhibition of the proteolytic activity of proteinase H by EDTA and deglycosylation prevented the digestion of Bβ fibrinogen but did not prevent the clotting of fibrinogen.

As to its fibrinogen-clotting activity, proteinase H behaves in a manner similar to Venzyme and the fibrinogen-clotting enzyme found in *A. halys* venom

by preferentially releasing FPB, with the weak release of FPA (STOCKER AND MEIER, 1988; DYR *et al.*, 1989). Proteinase H also has several features in common with cathepsin-L2 from *Fasciola hepatica*, the only other non-serine proteinase reported to cause fibrinogen-clotting (DOWD *et al.*, 1995). Neither proteinase H nor cathepsin-L2 are serine-proteases, fibrin anti-polymerants do not inhibit the clot-formation catalyzed by either of these agents, and both digest at least one of the three fibrinogen subunits. Also, the physical characteristics of the fibrin clot formed by both of these proteases is similar. In both cases the clot is less stable than that formed by thrombin, collapsing to form a white precipitate upon vigorous vortexing. Also, the ring formed by both of these agents in a fibrinogen-agarose plate assay is opaque and dense as opposed to the hazy ring formed by thrombin. The clots formed by EDTA-treated or deglycosylated proteinase H are physically indistinguishable from the thrombin-formed clots and electrophoretic analysis of these clots reveals that the α subunit is not digested. These findings suggest that the physical difference between the thrombin-formed and proteinase H-formed clots are due to this digestion of the $B\beta$ fibrinogen subunit.

Proteinase H is devoid of esterase activity on the substrates BAEE and TAME (KURECKI AND KRESS, 1985). A fibrinogen-clotting enzyme from *Dispholidus typus* (boomslang) venom also failed to cleave BAEE when tested (HIESTAND AND HIESTAND, 1979). Since esterase activity is not a prerequisite for fibrin clot formation, the fibrinogen-clotting activity of proteinase H is probably not due to any hidden or residual esterase activity.

As mentioned above, proteinase H is the first snake venom toxin reported to possess both a metalloproteinase-dependent hemorrhagic activity as well as fibrinogen-clotting activity. This does not exclude other fibrinogen-clotting and fibrino(geno)lytic enzymes as hemorrhagic toxins. Of the numerous snake

venom fibrinogen-clotting enzymes isolated to date none have been found to be hemorrhagic. However, only one such enzyme, ancrod, has been tested for hemorrhagic activity *in vivo* (HATTON, 1973). Conversely, of the many non-fibrinogen-clotting fibrinogenolytic snake venom enzymes that have been isolated several have also been found to be hemorrhagic (CIVELLO *et al.*, 1983; NIKAI *et al.*, 1985; KOMORI *et al.*, 1985; NIKAI *et al.*, 1986; TU, 1988). Each of these enzymes was isolated primarily as a hemorrhagic toxin and was later characterized as being fibrinogenolytic *in vitro*. It is surprising that of the many snake venom enzymes that affect hemostatic components of the blood, so few have been tested for both fibrinogen-clotting activity and hemorrhagic activity. As such, it seems premature to conclude that fibrinogen-clotting enzymes can not also be hemorrhagic.

In conclusion, our findings indicated that proteinase H is a potent hemorrhagic toxin that also possesses fibrinogen-clotting activity. Proteinase H releases fibrinopeptide B to induce fibrinogen-clotting and also digests the Bβ subunit of fibrinogen.

Acknowledgments: The authors thank Drs. Paulo Melo and Heloisa S. S. Araujo for their help in the preparation of this manuscript and Dr. Thomas J. Mohn, Dr. Robert Paul Schwab and Mr. Kevin Stepaniuk for critically reading it.

This study was conducted in partial fulfillment of the requirements for the Ph. D. degree at Oklahoma State University for S. G. Anderson and was supported by a Ford Foundation Predoctoral Research Fellowship (S. G. A.) and a Public Health Service Grant 5 R01 AI26923-07 from the National Institute of Allergy and Infectious Diseases (C. L. O.).

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CHAPTER III

PARTIAL CHARACTERIZATION AND THE ROLE OF GLYCOSYL MOIETIES OF THE HEMORRHAGIC TOXIN, PROTEINASE H, FROM *CROTALUS ADAMANTEUS* VENOM

ABSTRACT

Proteinase H was purified using open column and HPLC liquid chromatographic procedures modified from the method first reported by KURECKI and KRESS in 1985. The protein gave a positive reaction for carbohydrate when stained with periodic acid-Schiff's reagent. A decrease in the molecular weight of proteinase H as estimated by reducing SDS-PAGE was observed following removal of sugar moieties with the peptide *N*-glycosidase from *Flavobacterium meningosepticum* (PNGaseF). The nature of the glycosylation was determined by lectin affinity blotting against the toxin with *Datura stramonium* agglutinin (DSA) which is specific for glycosyl residues containing the disaccharide galactose-β(1-4)-*N*-acetylglucosamine in complex and hybrid *N*-glycans (CROWLEY *et al.*, 1984). The role of sugar moieties in the proteolytic and hemorrhagic activities of proteinase H were studied. Removal of sugar moieties by PNGaseF or their oxidation by 0.5 M sodium periodate totally abolished the hemorrhagic and proteolytic

activity of the toxin. Removal of sialic acid residues by neuraminidase from *Vibrio cholerae* had no effect on hemorrhagic activity but resulted in about a 90% decrease in proteolytic activity on Azocoll™. These data indicate that the glycosyl moieties of proteinase H, in their intact ring formations, are essential to the enzymatic and hemorrhagic activity of the toxin.

INTRODUCTION

Approximately 80 hemorrhagic toxins have been isolated from venoms of the various poisonous snakes around the world (MARSH, 1994; BJARNASON and FOX, 1994). Hemorrhagic toxins are responsible for a large portion of the local tissue damage that is often associated with snake bite envenomations, and contribute to or in severe cases even cause death. Many of the hemorrhagic toxins thus far isolated have been at least partially characterized (BJARNASON and FOX, 1988). As a group, snake venom hemorrhagic toxins are zinc-dependent metalloproteinases and many of them are fibrino(geno)lytic. Also, snake venom hemorrhagic toxins, in general, are known to be glycoproteins with a varied degree of glycosylation (KURECKI and KRESS, 1985; LI *et al.*, 1993; SÁNCHEZ *et al.*, 1987).

While many of the physicochemical and biochemical traits of hemorrhagic toxins have been studied, very little is known about the glycosyl moieties of snake venom hemorrhagic toxins. In most cases neither the identities nor the functions of these glycosyl residues are known. Recently, two hemorrhagic glycoproteins from the venom of *Crotalus viridis viridis* were isolated and the nature of their glycosylation investigated (LI *et al.*, 1993). In this study it was reported that the two hemorrhagic toxins both contained Gal-β(1-4)GlcNAc side

chains which could be removed with PNGaseF, thus blocking the enzymatic and hemorrhagic activities of the toxins.

Proteinase H is a hemorrhagic metalloproteinase that was first isolated from *Crotalus adamanteus* venom by KURECKI and KRESS in 1985. At that time it was reported that this toxin was the sole agent responsible for the considerable hemorrhagic capacity of *C. adamanteus* venom and that it carried a large degree of glycosylation. In the present study we report the characterization of the glycosyl moieties of proteinase H and investigate their role in the action of the toxin *in vivo* and *in vitro*. We demonstrate that the glycosyl moieties of such a toxin may be directly involved in the action of the toxin.

MATERIALS AND METHODS

Venom and reagents

Crude *C. adamanteus* (Eastern diamondback rattlesnake) venom from a single pool of snakes from Florida was purchased in lyophilized form from Biotoxins, Inc. (St. Cloud, FL, U. S. A.). *N,N*-dimethylated casein (DMC) was purchased from Sigma Chemical Co. (St. Louis, MO, U. S. A.). Azocoll™ and 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPS) were purchased from Calbiochem (La Jolla, CA, U. S. A.). PNGaseF from *Flavobacterium meningosepticum* and neuraminidase (NMD) from *Vibrio cholerae* were purchased from Boehringer Mannheim Corp. (Indianapolis, IN, U. S. A.).

Purification of proteinase H

The hemorrhagic toxin, proteinase H, was isolated from *Crotalus adamanteus* venom in four liquid chromatographic steps as described in Chapter II. Crude venom was separated by open column gel filtration chromatography

on a Sephacryl S-200 HR column followed by DEAE anion exchange HPLC and sulfopropyl cation exchange HPLC. The formation of a single band on SDS-PAGE stained with silver nitrate demonstrated the homogeneity of the toxin.

Amino acid analysis

Amino acid analysis was performed in the Molecular Biology Resource Facility at the William K. Warren Medical Research Institute, University of Oklahoma Health Sciences Center under the direction of Dr. Ken Jackson. Samples were subjected to hydrochloric acid hydrolysis with 6N HCl at 110°C for 24 hours. Additional samples underwent performic acid hydrolysis for the determination of 1/2 cystine. Amino acid analysis was performed by cation exchange chromatography (SPACKMAN *et al.*, 1958). Elution was accomplished by a two buffer system, using 0.2 N sodium citrate (pH 3.28) and 1.0 M NaCl (pH 7.4). Amino acids were detected by on-line post-column reaction with ninhydrin (Trione, Pickering Laboratories, Inc.). Derivatized amino acids were quantitated by their absorption at a wavelength of 570 nm, except for glutamic acid and proline, which are detected at a wavelength of 440 nm. The procedure was performed on a totally automated Beckman system Gold model 126 HPLC amino acid analyzer. Tryptophan was determined spectrophotometrically.

Determination of hemorrhagic activity

Hemorrhagic activity of samples was determined by the intradermal injection of 100 µl of treatment solution into the backs of white, female CD-1 mice (24-26 g), (n=3). After three hours the mice were killed by cervical dislocation and the size of the hemorrhagic spot surrounding the injection site was measured as described by OWNBY *et al.* (1984).

Polyacrylamide gel electrophoresis and electroblotting

Crude *C. adamanteus* venom (1.0 µg), proteinase H (0.1 or 0.05 µg), and asialofetuin (0.1 µg) used as a control for glycan differentiation were subjected to SDS-PAGE. Samples were prepared in sample buffer containing 62.5 mM Tris-HCl (pH 8.0), 2% SDS, 2% β-mercaptoethanol and 20% glycerol with bromophenol blue as a running front marker, and were heated at 100°C for 5 minutes. Electrophoresis was carried out using a Pharmacia PhastSystem electrophoresis unit and either 12.5 % T homogeneous or 8-25 % T gradient acrylamide gels. Samples were applied to the gels and electrophoresis was carried out for 65 V•hr for 12.5 % homogeneous gels or 80 V•hr for gradient gels according to the manufacturer's instructions as described in Pharmacia PhastSystem Technical Bulletin No. 110. Following electrophoresis gels were either stained or transferred to PVDF membranes for immunoblotting. Gels were stained with Coomassie blue or silver nitrate where indicated. Molecular weights were estimated using SigmaMarker Wide Molecular Weight Range marker kit No. M-4038 (Sigma Chemical Co., St. Louis, MO, U. S. A.).

Proteins were transferred to nitrocellulose membranes by semi-dry electroblotting procedure using the PhastTransfer™ semi-dry transfer apparatus (Pharmacia Biotech, Piscataway, NJ, U. S. A.) as described in the manufacturer's instructions.

Determination of carbohydrate content and percent glycosylation

Glycosylation was first determined by staining of SDS-PAGE gels with periodic acid-Schiff stain (KAPITANY and ZEBROWSKI, 1973). The toxin was treated with PNGaseF and then separated by electrophoresis (LI *et al.*, 1993) to determine the carbohydrate mass content as well as the linkage of the residues.

The molecular weight of the deglycosylated toxin and the non-treated toxin were estimated by comparison with molecular weight standards (LI *et al.*, 1993).

Glycan differentiation

Identification of the glycan linkage was determined by the susceptibility of the sugar moieties to hydrolysis by PNGaseF. The nature of the glycan moieties involved was determined using a glycan differentiation kit (Boehringer Mannheim Corporation, Indianapolis, IN, U. S. A.) following the manufacturer's instructions. Briefly, the toxin sample was first subjected to preliminary analysis by dot-blotting in which 1 μg in 1 μl of Tris-buffered saline (pH 7.5) was vacuum blotted onto a PVDF membrane and analyzed to determine the lectin specificity for the toxin as described below. Following the preliminary specificity determination using the dot-blotting technique, the samples were electrophoresed and transferred to blotting membranes as described above. The membranes were incubated with blocking solution overnight at 4°C and then reacted with one of the following five different digoxigenin-labeled lectins: *Galanthus nivalis* agglutinin (recognizes terminal Man-α(1-3)Man, Man-α(1-6)Man or Man-α(1-2)Man), *Sambucus nigra* agglutinin (specific for Sia-α(2-6)Gal), *Maackia amurensis* agglutinin (specific for Sia-α(2-3)Gal), peanut agglutinin (recognizes core disaccharide Gal-β(1-3)GlcNAc), and *Datura stramonium* agglutinin (DSA) (recognizes Gal-β(1-4)GlcNAc in complex and hybrid N-glycans or O-glycans and GlcNAc in O-glycans). Following the incubation with the lectins the membranes were washed and then incubated for 1 hour at room temperature with sheep polyclonal anti-digoxigenin Fab fragments conjugated with alkaline phosphatase. Following a final wash the membranes were developed using a solution of 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoyl-phosphate dissolved in dimethylformamide.

Effect of EDTA and carbohydrate removal on hemorrhagic activity of proteinase H

The effects of zinc-chelation by EDTA and deglycosylation of proteinase H with PNGaseF on the hemorrhagic activity of proteinase H were determined. Briefly, five micrograms of proteinase H in 25 µl of 0.01 M Tris-HCl (pH 7.2) were mixed with an equal volume of 5 mM EDTA (pH 8.6) and incubated at 4°C overnight. For deglycosylation the same amount of toxin was mixed with 25 ml of PBS containing 0.4 units of PNGaseF and incubated at 37°C overnight. After incubation hemorrhagic activity was measured as described above.

Effect of removal of sialic acid residues on proteolytic and hemorrhagic activities of proteinase H

Proteinase H in aqueous solution (5µg in 10 µl) and *Vibrio cholerae* neuraminidase (0.05 U in 50 µl of sodium phosphate buffer, pH 7.2) were made up to 100 µl in buffer and incubated overnight at 37°C for removal of sialic acid residues. Following this treatment the sample was tested for proteolytic activity on *N,N*-dimethylated casein and Azocoll™ and for hemorrhagic activity as described above.

Proteolytic activity of proteinase H on N,N-*dimethylated casein*

Proteinase H (5.0 µg) was treated with either EDTA, PNGaseF or neuraminidase or left untreated as a control. Each sample was incubated in 1.5 ml microcentrifuge tubes as described above. Following this incubation period the proteolytic activity of the incubation mixture was determined using *N, N*-dimethylated casein (DMC) as a substrate (JOHNSON and OWNBY, 1993). DMC (0.5 g) was dissolved in 100 ml of 5 mM MOPS buffer (pH 7.2). Samples to be tested (50 µl) were transferred to test tubes (n = 3) and 1 ml of the DMC solution

added to each tube. Tubes containing 50 µl of MOPS buffer were used as negative controls and 1 µg of pronase in 50 µl of MOPS buffer was used as a positive control. The tubes were incubated for 30 minutes in a 37°C water bath to allow proteolysis to occur. The proteolytic reactions were stopped by heating the tubes 5 minutes at 100°C. After heating, 1 ml of 0.4 M NaH_2CO_4 buffer (pH 8.5) and 1 ml of 0.1 % TNBS was added to each tube for the color development reaction. The tubes were incubated for 20 minutes in a 50°C water bath to allow for color development. The color development reactions were stopped by the addition of 1 ml of 10% SDS and 0.5 ml of 1.0 N HCl to each tube. 200 µl from each tube was transferred to a well in a Linbro® (Flow Laboratories, McLean, VA, U. S. A.) microtiter plate. The OD at 340 nm of each sample was determined using a Thermomax ELISA plate reader (Molecular Devices, Menlo Park, CA, U. S. A).

Proteolytic activity of proteinase H on Azocoll™

Proteinase H (5.0 µg) was treated with either EDTA, PNGaseF or neuraminidase or left untreated as a control. Each sample was incubated in 1.5 ml microcentrifuge tubes as described above. After the incubation period 50 µl of Tris buffer was added to each tube. Azocoll™ (0.5 g) was then suspended in 15 ml of 10 mM Tris buffer (pH 7.0) and 100 µl of this suspension added to each of the tubes. One tube containing Tris buffer and Azocoll™ suspension only and tubes containing EDTA, PNGaseF or neuraminidase without proteinase H were used as negative controls. Samples were incubated for 1 hour at 37°C on an orbital shaker at 250 rpm. After incubation the samples were centrifuged at 1000 X *g* for 15 minutes then 100 µl of the supernatant solution from each sample was transferred to the wells of a Linbro® (Flow Laboratories, McLean, VA, U. S. A.) microtiter plate. The absorbance of the supernatant solution was

read at 520 nm using a Thermomax ELISA plate reader (Molecular Devices, Menlo Park, CA, U. S. A.).

Statistics

The data from each experimental group were subjected to a two-way analysis of variance (ANOVA). Variances were expressed as standard errors of means. Differences between treatments and controls were calculated and analyzed using the Tukey method of multiple comparisons. Differences were considered significant if $p < 0.05$.

RESULTS

Purification of proteinase H

From 2.0 g of crude venom a homogeneous fraction containing 10.5 mg of total protein was obtained. Homogeneity of this material was determined as described above and is shown in Fig. 1A.

Estimation of molecular weight following deglycosylation

Proteinase H gave a strong positive reaction with the PAS stain on an SDS-PAGE gel. Deglycosylation of proteinase H with PNGaseF resulted in complete abolition of the positive reaction with PAS stain reagents. Additionally, as seen in Fig. 1B, treatment of the toxin with 0.4 units of PNGaseF resulted in an increase in the electrophoretic mobility of the toxin. The molecular weights estimated by comparison with molecular weight standards were 85.7 kD for the non-treated toxin and 50.6 kD for the treated toxin. Removal of sialic acid residues by treatment with neuraminidase had no effect on the electrophoretic mobility of proteinase H, indicating that sialic acid residues, if any were present,

make no significant contribution to the carbohydrate mass content of proteinase H.

Glycan differentiation

Blotting of the native toxin and deglycosylated toxin was performed as described above in methods. In the preliminary dot blotting experiment the toxin solution reacted only with digoxigenin-labeled DSA. Following separation on SDS-PAGE reaction of the toxin with DSA resulted in the formation of a single strong band (Fig. 2) indicating the presence of disaccharide galactose-β(1-4) *N*-acetylglucosamine glycosyl residues.

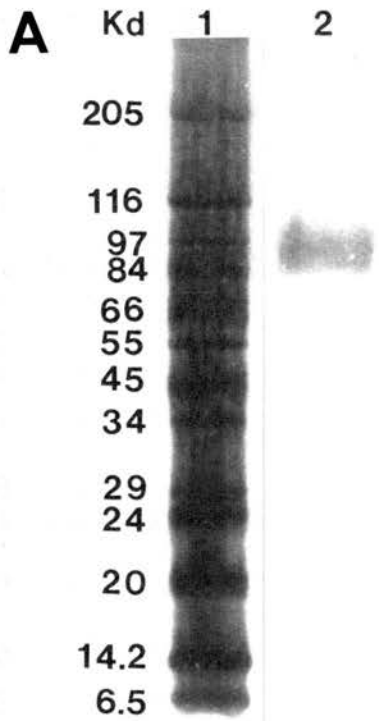
Amino acid composition

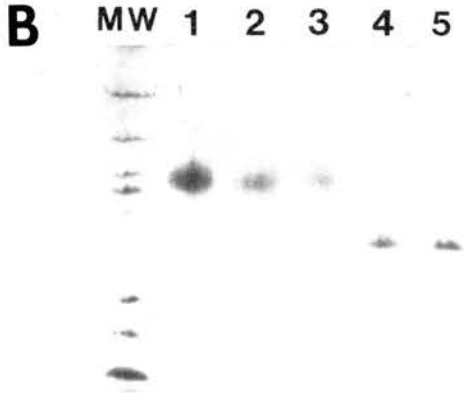
The amino acid composition of deglycosylated proteinase H was determined as described above (Table 1). The minimum molecular weight calculated from the amino acid composition data was 49.4 kD.

Effect of EDTA and carbohydrate removal on hemorrhage

The effects of EDTA, PNGaseF and neuraminidase on the hemorrhagic activity of proteinase H are shown in Table 2. EDTA (2.5 mM final concentration) significantly inhibited the hemorrhage induced by proteinase H. The occurrence of a small amount of hemorrhage induced by EDTA alone made it somewhat difficult to determine whether the toxin still exhibited any hemorrhagic capability at all. However, since the hemorrhagic indices from mice treated with EDTA alone were not significantly different ($p < 0.05$) from those of mice treated with proteinase H plus EDTA, the hemorrhagic activity of proteinase H was completely inhibited. Removal of the carbohydrate moieties by 0.4 units of PNGaseF or their oxidation by 0.5 M $NaIO_4$ both resulted in the

FIG. 1. SDS-PAGE OF HOMOGENEOUS PROTEINASE H. Samples of proteinase H (100 ng) were separated by denaturing SDS-PAGE on 8-25 % T gradient acrylamide gels. (A) Lane 1, molecular weight markers; Lane 2, proteinase H. (B) Lane 1, untreated control proteinase H; Lane 2, proteinase H + 5 mM EDTA incubated overnight at 37°C; Lane 3, proteinase H incubated overnight at 37°C, Lane 4, proteinase H + 5 mM EDTA and 0.4 U PNGaseF incubated overnight at 37°C; Lane 5, proteinase H + 0.4 U PNGaseF incubated overnight at 37°C. Visualization was performed by staining with silver nitrate.


A
Kd
1
2
205
116
97
84
66
55
45
34
29
24
20
14.2
6.5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1
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4
5

FIG. 2. LECTIN BLOTTING OF PROTEINASE H. Following separation by SDS-PAGE proteins were transferred to PVDF membranes for blotting against digoxigenin-labeled DSA. Lane 1, crude *C. adamanteus* venom (1.0 μg); Lane 2, proteinase H (0.1 μg); Lane 3, asialofetuin control (0.1 μg); Lane 4, deglycosylated proteinase H (0.1 μg); Lane 5, PNGaseF control (0.008 U).


1 2 3 4 5

TABLE 1 AMINO ACID COMPOSITION OF PROTEINASE H

Amino Acid	Residues per molecule
Aspartic acid	20
Threonine	24
Serine	24
Glutamic acid	35
Proline	15
Glycine	28
Alanine	20
Valine	19
Isoleucine	30
Leucine	30
Tyrosine	14
Phenylalanine	11
Lysine	14
Histidine	11
Arginine	12
Methionine	10
1/2 Cystine*	33
Tryptophan§	5
Total	355
Formula weight	49,400

*Determined as cysteic acid

§Determined spectrophotometrically

TABLE 2 INHIBITION OF PROTEINASE H-INDUCED HEMORRHAGE BY TREATMENT WITH DIFFERENT REAGENTS

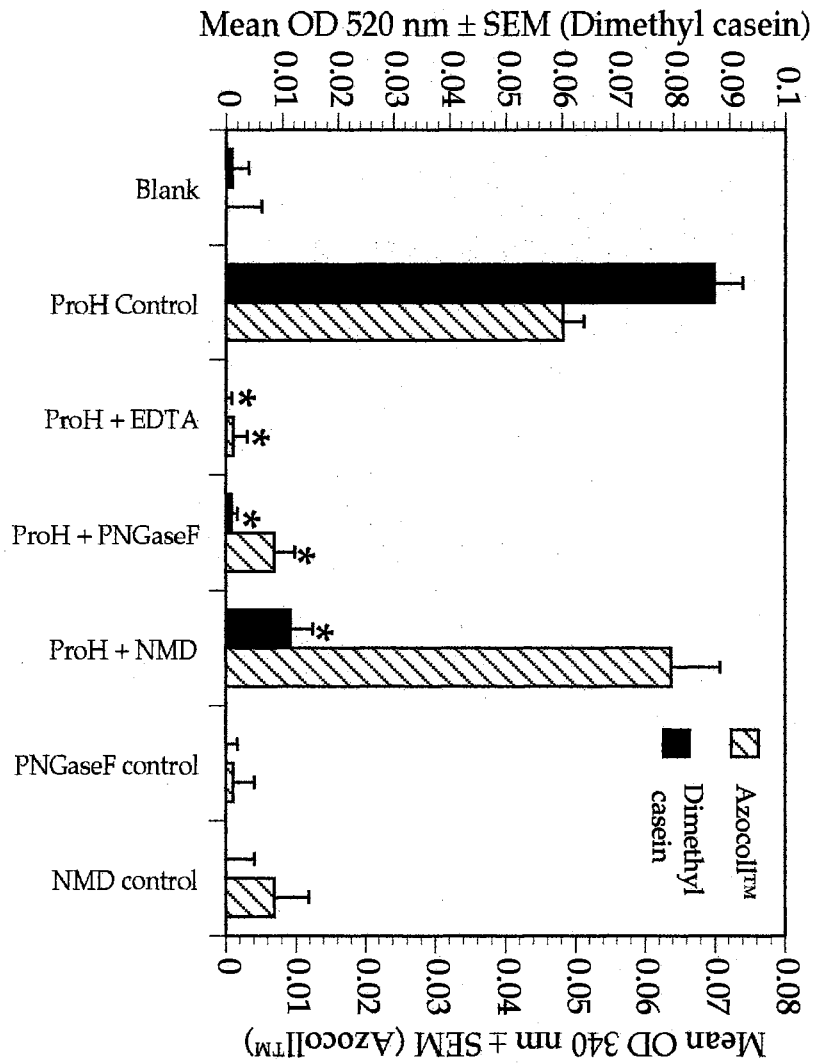
Treatment	Mean Hemorrhagic Index*	% Inhibition
ProH	238 ± 12	0
EDTA	28 ± 7	88 ± 6
ProH + EDTA	33 ± 13	86 ± 6
ProH + PNGaseF	0 ± 0	100 ± 0
ProH + NMD	209 ± 11	12 ± 5

Proteinase H was treated with the above reagents as described in the methods section of this paper

*Hemorrhagic indices were calculated as the products of perpendicular diameters (in mm) of hemorrhagic spots

Results are expressed as means of calculated hemorrhagic indices ± SEM (n=3)

FIG. 3. INHIBITION OF PROTEOLYTIC ACTIVITY OF PROTEINASE H ON AZOCOLL™ AND N,N-DIMETHYLATED CASEIN (DMC) BY PRE-TREATMENT WITH PNGASEF, NEURAMINIDASE OR EDTA. 50 μg of Proteinase H in 10 mM Tris buffer (pH 8.5) was treated with PNGaseF, neuraminidase (NMD), or EDTA and then tested for proteolytic activity on Azocoll™ and N,N-dimethylated casein as substrates as described in methods. Values are expressed as mean OD 520 ± SD (n = 3) and OD 340 nm ± SEM (n = 3). *: significantly different from proteinase H control ($p<0.05$).


Mean OD 520 nm ± SEM (Dimethyl casein)
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.1
Blank
ProH Control
ProH + EDTA
ProH + PNGaseF
ProH + NMD
PNGaseF control
NMD control
*
Dimethyl casein
Azocoll™
Mean OD 340 nm ± SEM (Azocoll™)
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08

complete inhibition of the hemorrhagic action of the toxin. Desialylation with neuraminidase had no effect on the hemorrhagic activity of proteinase H.

Inhibition of proteolytic activity on N,N-*dimethylated casein and Azocoll™*

Treatment of proteinase H with either EDTA abolished all detectable proteolytic activity on Azocoll™ and *N,N*-dimethylated casein. Treatment with PNGaseF inhibited the proteolytic digestion of Azocoll™ and *N,N*-dimethyl casein by 99 ± 2% and 86 ± 12% respectively (Fig. 3). Treatment of proteinase H with neuraminidase resulted in a decrease in the proteolysis of Azocoll™ by approximately 87 ± 9% (Fig. 3). The desialylated proteinase H showed an enhanced proteolysis of *N,N*-dimethylated casein, having an activity that was 24 ± 12% greater than that of the proteinase H control group.

DISCUSSION

The mechanism of action of snake venom hemorrhagic toxins is as yet poorly understood. It is generally accepted that their physiological action is due to the proteolysis of capillary basement membrane proteins (BARAMOVA *et al.*, 1989; BARAMOVA *et al.*, 1991). A rather common characteristic of snake venom proteins is that of a varying degree of glycosylation (GOWDA and DAVIDSON, 1992). Many of the snake venom hemorrhagic toxins isolated thus far have been shown to be glycoproteins (KURECKI and KRESS, 1985; LI *et al.*, 1993; SÁNCHEZ *et al.*, 1987), but relatively little is known about the nature or role of their constituent carbohydrate moieties. It was recently demonstrated that the carbohydrate moieties of some snake venom hemorrhagic toxins may be involved in their hemorrhagic activity (LI *et al.*, 1993).

The evidence presented in this study indicates that proteinase H is heavily glycosylated. This is indicated by the decrease in electrophoretically estimated molecular weight. Anomalies in the electrophoretic mobility of such heavily glycosylated proteins prevent an accurate estimation of the molecular weight of the native, glycosylated protein, thus the carbohydrate mass content cannot be accurately determined.

Glycosylation is an important aspect of the function of many proteins, although ideas on the exact role of glycosylation vary widely. Glycosylation is generally thought to play one of three non-specific roles. Glycosylation may (1) protect the toxin from proteolysis, (2) provide molecular stability, or (3) provide the proper surface charge for molecular activity (WEST, 1986). Glycosylation may also play a very specific role such as in intracellular routing of secretory proteins and possibly in recognition (WEST, 1986). Glycosylation has been shown to be necessary for the binding of certain glycoproteins to their substrate, and the binding of the glycoprotein can be competitively inhibited by the isolated glycan moieties of that glycoprotein (SWANSON and KUO, 1994). Some studies also suggest that sugar moieties may play a role in the cellular metabolism of certain substances such as LDL (FUJIOKA *et al.*, 1994).

The loss of activity following deglycosylation suggests that the glycosyl moieties of proteinase H play an important role in the activity of the enzyme. The changes in the electrophoretic mobility of PNGaseF-treated proteinase H both in the presence of EDTA and without EDTA are identical. Because all detectable proteolytic activity of proteinase H is inhibited by EDTA it can be concluded that no autoproteolysis is occurring to which the decrease in apparent molecular weight can be attributed. Additionally, reducing SDS-PAGE of PNGaseF-treated proteinase H results in the formation of a single band, also indicating that no proteolysis of the toxin has occurred. This then rules out the

role of glycans in protection of proteinase H from autoproteolysis, but not necessarily from proteolytic degradation by heterologous proteinases. It can be concluded that under the experimental conditions described above the loss of the hemorrhagic and proteolytic activity of proteinase H must be attributed directly to the removal of its glycosyl moieties. A similar study on the enzymatic activity of peanut peroxidase (PRX) found that the loss in activity of PRX was due to conformational changes in the molecule related to the loss of heme and calcium from the enzyme (VAN HUYSTEE and WAN, 1994). Earlier studies on the role of zinc ions in the enzymatic activities of snake venom metalloproteinases determined that removal of zinc from fibrolase by chelating agents (i.e. EDTA) resulted in changes in the three-dimensional conformation of the enzyme, specifically, the uncoiling of the α helical structure of the domain containing the putative active site of fibrolase (PRETZER, *et al.*, 1992).

Additionally, the glycosyl moieties may be involved in more than just maintenance of the structural integrity of the protein and could be involved directly in the interaction of the toxin with substrate and/or cellular target site(s). Carbohydrate moieties are known to be very important in cell adhesion strategies, especially those involving selectins. Selectin ligands, such as GlyCAM-1, are glycoproteins that have a high mass content of carbohydrates, a characteristic that seems to be a common motif among large snake venom hemorrhagic toxins (LEVY *et al.*, 1994).

Glycosylation also plays an important role in maintenance of hemostasis as evidenced by the inhibition of platelet aggregation by disintegrins that interact with the $\alpha II_b\beta 3$ integrin found on the surface of platelets (LU *et al.*, 1993; TAKEYA *et al.*, 1993). It has been proposed that snake venom hemorrhagic toxins and disintegrins may be derived from the same precursor molecules by proteolytic degradation and that large hemorrhagic toxins may still contain the

disintegrin subunit found in the original precursor molecule (KINI and EVANS, 1992). There is also evidence that snake venom disintegrins and plant lectins share sequence similarities (KIELISZEWSKI *et al.*, 1994). This supports the idea that glycosylation of ligands and carbohydrate identification are important in the function of snake venom hemorrhagic toxins. Based on these ideas it seems likely that the glycosylation of snake venom hemorrhagic toxins plays an important role in the pathogenesis of hemorrhage induced by such toxins.

The findings of the current study support the idea regarding the activity of the hemorrhagic toxin proteinase H and suggest that intact carbohydrate moieties of proteinase H maintain the three-dimensional stability of the toxin. Further study is necessary to determine what, if any, role glycosyl moieties may play in toxin specificity or binding by serving as a ligand or "docking structure".

Acknowledgments: The authors thank Drs. Paulo Melo and Heloisa S. S. Araujo for their help in the preparation of this manuscript and Thomas J. Mohn, Robert Paul Schwab and Kevin Stepaniuk for critically reading this manuscript.

This study was conducted in partial fulfillment of the requirements for the Ph. D. degree at Oklahoma State University for S. G. Anderson and was supported by a Ford Foundation Predoctoral Research Fellowship (S. G. A.) and a Public Health Service Grant 5 R01 AI26923-07 from the National Institute of Allergy and Infectious Diseases (C. L. O.).

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CHAPTER IV

PATHOGENESIS OF HEMORRHAGE INDUCED BY PROTEINASE H FROM EASTERN DIAMONDBACK RATTLESNAKE (*CROTALUS ADAMANTEUS*) VENOM

ABSTRACT

The pathogenesis of hemorrhagic effects of a purified hemorrhagic toxin, proteinase H from *Crotalus adamanteus* venom, was studied. Female, white CD-1 mice were injected intramuscularly with sublethal doses of the hemorrhagic toxin and tissue samples were obtained at 10 minutes, 1, 3 and 24 hours following injection. Severe local hemorrhage was observed grossly within 10 minutes. Hemorrhage were observed primarily involved the microvasculature within the connective tissue of the skeletal muscle and within adjacent adipose tissue. Many larger vessels were congested with erythrocytes and platelets. By 3 hrs inflammatory cell infiltration was observed and necrosis of some muscle cells was evident. Transmission electron microscopy showed that the capillary endothelium was ruptured, leading to hemorrhage *per rhexis*. Capillary basal laminae were disorganized and often wholly or partially absent.

INTRODUCTION

Hemorrhage is a consistent symptom in snake bite envenomation and a major contributor to the overall pathology in many cases. Many venoms from snakes of the family Viperidae have been found to contain at least one or more hemorrhagic proteins. In most cases a single venom contains more than a single hemorrhagic toxin. Venom from *Crotalus atrox* (Western diamondback rattlesnake) contains no less than seven distinct hemorrhagic toxins (BJARNASON and TU, 1978; NIKAI *et al.*, 1984; NIKAI *et al.*, 1985; BJARNASON *et al.*, 1988). *C. adamanteus* venom, however, has been found to contain a single hemorrhagic metalloproteinase (KURECKI and KRESS, 1985; Chapter II).

Of the many known snake venom hemorrhagic toxins, most have been well characterized biochemically. In the last decade investigations of their biological mode of action in the induction of hemorrhage have been undertaken. Most studies have utilized light and electron microscopic techniques to study the pathological effects of hemorrhagic toxins on the vasculature *in vivo* (QUEIROZ *et al.*, 1985; OWNBY and GEREN, 1987; OWNBY *et al.*, 1990; JOHNSON and OWNBY, 1993). Other studies have characterized the effect of hemorrhagic toxins from *Bothrops asper* venom on cultured endothelial cells (MOREIRA *et al.*, 1994; BORKOW *et al.*, 1994, 1995). The purpose of this study was to define the *in vivo* effects of proteinase H on the murine vasculature following intramuscular injection. Using light and electron microscopy the pathogenesis of the hemorrhage induced by the toxin was thus determined.

MATERIALS AND METHODS

Purification of proteinase H

Crude *Crotalus adamanteus* venom (lot No. CA/87A) was obtained in lyophilized form from Biotoxins, Inc. (St. Cloud, FL, U. S. A.) and stored desiccated at -20°C until use. The venom was fractionated according to the method described by KURECKI and KRESS (1985), with minor modifications. Specifically, in the last step of the fractionation a Waters DEAE-5PW semi-preparative HPLC anion exchange column was used in lieu of the open column DEAE-Sepharose used by Kurecki and Kress. Homogeneity of the purified toxin was demonstrated by the formation of a single band on a SDS-PAGE gel stained with silver nitrate and by analytical C18 reversed-phase HPLC.

Sample preparation and microscopy

Experimental female mice (CD-1, Charles River) weighing 22-24 g were injected with 100 µl of a solution containing a final dose of either 1 or 5 µg toxin/g mouse weight. Control mice were injected with 100 µl of physiological saline. All mice were injected intramuscularly in the lateral aspect of the right thigh (biceps femoris muscle) and were killed by cervical dislocation at either 10 minutes, 1, 3, or 24 hours following injection. Muscle samples were taken from the medial aspect of the injected thigh (gracilis and semimebranous muscles) to avoid sampling regions that could have possibly suffered mechanical damage during the injection. A total of twenty-one mice were used in three separate experiments (n=3).

After sampling the tissue was immediately processed for embedding in either paraffin or plastic resin. For embedding in paraffin tissue samples were fixed in Bouin's solution overnight and then washed overnight in three changes

of 70 % ethanol. The tissue was then embedded in paraffin and 3 μm sections were taken, mounted with Permount on glass slides, and stained with hematoxylin and eosin for viewing. Tissue samples to be embedded in plastic resin were fixed initially in 2 % glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) and embedded in Polybed resin (Polysciences, Warrington, PA, U. S. A.) as previously described (OWNBY and GEREN, 1987). Thick (0.7 μm) sections were taken from blocks of the plastic-embedded tissue using a Sorvall MT-5000 ultramicrotome with glass knives and were stained with Mallory's trichrome for light microscopy. Blocks were selected for sectioning by random selection (n=5).

Thin sections (70-90 nm) of selected areas of the plastic embedded blocks were taken for electron microscopy with a Sorvall MT-6000 ultramicrotome using a diamond knife. Sections were picked up on copper grids (200 mesh) and stained with methanolic uranyl acetate and lead citrate, observed and photographed with a JEOL 100 CX TEMSCAN electron microscope at an accelerating voltage of 80 KV.

RESULTS

The micrographs that are pictured in this section demonstrate observations that were typical of all tissue blocks that were sectioned. No hemorrhage was apparent by either gross or light microscopic observation following IM injection of the physiologic saline vehicle (Fig. 1). Electron microscopic analysis of vascular structures in these control samples confirmed normal tissue fine structure.

Gross observation following intramuscular injections of 1 μg and 5 μg proteinase H per gram of mouse weight revealed hemorrhage and edema surrounding the site of injection. No effort was made to quantitate this

hemorrhage. Within 3 hrs the injected limb was noticeably swollen and turgid and edema was evident upon dissection. By 24 hrs the tissue was very dark red and noticeably harder than the normal tissue.

Light microscopy of H & E stained paraffin sections revealed extensive areas of hemorrhage within the connective tissues within 10 min after injection of the toxin (Fig. 2A) compared with the control (Fig. 1). Figures 2B and 2C show that hemorrhage persists without resolution at 1 and 3 hrs. In figure 2C the first signs of inflammation are seen as neutrophils (PMNs) appear and are associated with necrotic muscle cells. Figure 2C shows visible muscle cell damage at a dose of 1μg/g and within 3 hrs while Fig. 3A shows similar damage within 1 hr at the 5 μg/g dose. Myofibrillar clumping and rupture of the sarcolemma can be seen in these necrotic muscle cells. Figure 2D shows persistent hemorrhage in the form of extravasated and hemolysed erythrocytes after 24 hrs and an increase in myonecrosis and PMN infiltration. Figure 3B shows extravasated and hemolysed erythrocytes and massive PMN infiltrate as well as myonecrosis 24 hrs after injection of the higher dose of proteinase H (5 μg/g).

Figure 4A shows an electron micrograph of an intact capillary 10 min after injection with proteinase H (5 μg/g). The capillary is comprised of two endothelial cells showing different degrees of damage. One endothelial cell is swollen, its plasma membrane is ruptured along the ablumenal surface and blebs project from its lumenal membrane. Figure 4B shows a higher magnification of these damaged endothelial cell membranes. The other endothelial cell pictured in Fig. 4A is not swollen but does contain some

FIG. 1. LIGHT MICROGRAPH OF CONTROL SKELETAL MUSCLE. Paraffin-embedded muscle tissue stained with H & E 24 hrs after intramuscular injection of physiological saline solution. (Bar = 100 μm.)

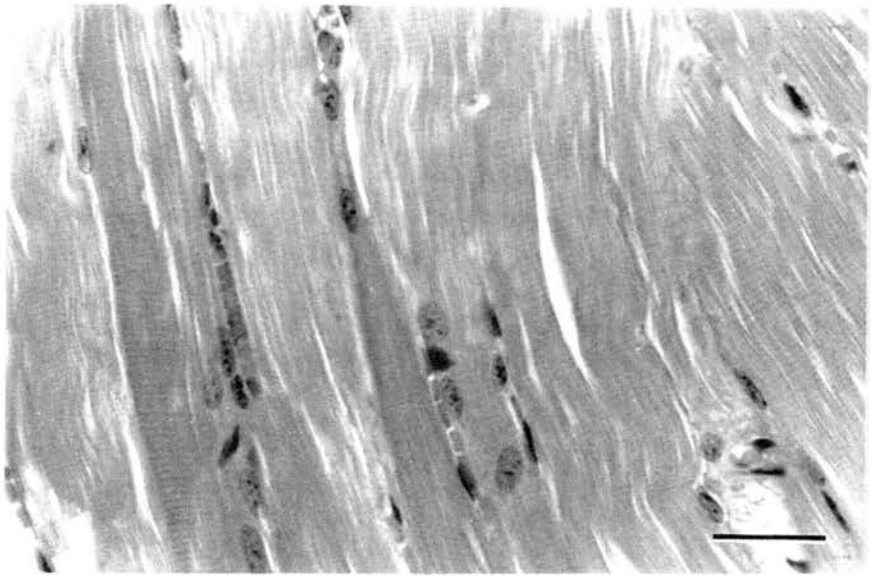


FIG. 2. LIGHT MICROGRAPHS OF SKELETAL MUSCLE AT VARIOUS TIMES AFTER INJECTION OF 1 μg/g PROTEINASE H. The micrographs show paraffin-embedded tissue stained with H & E. (A) Skeletal muscle tissue 10 min after injection showing extensive hemorrhage, H. (Bar = 50 μm.) (B) Muscle tissue showing areas of hemorrhage 1 hr after injection, H. (Bar = 40 μm.) (C) Skeletal muscle showing areas of hemorrhage, H, necrotic myocytes, N, and neutrophils, arrowheads, 3 hrs after injection. (Bar = 40 μm.) (D) Skeletal muscle 24 hrs after injection showing necrotic myocytes, N, and neutrophils, arrowhead. (Bar = 50 μm.)

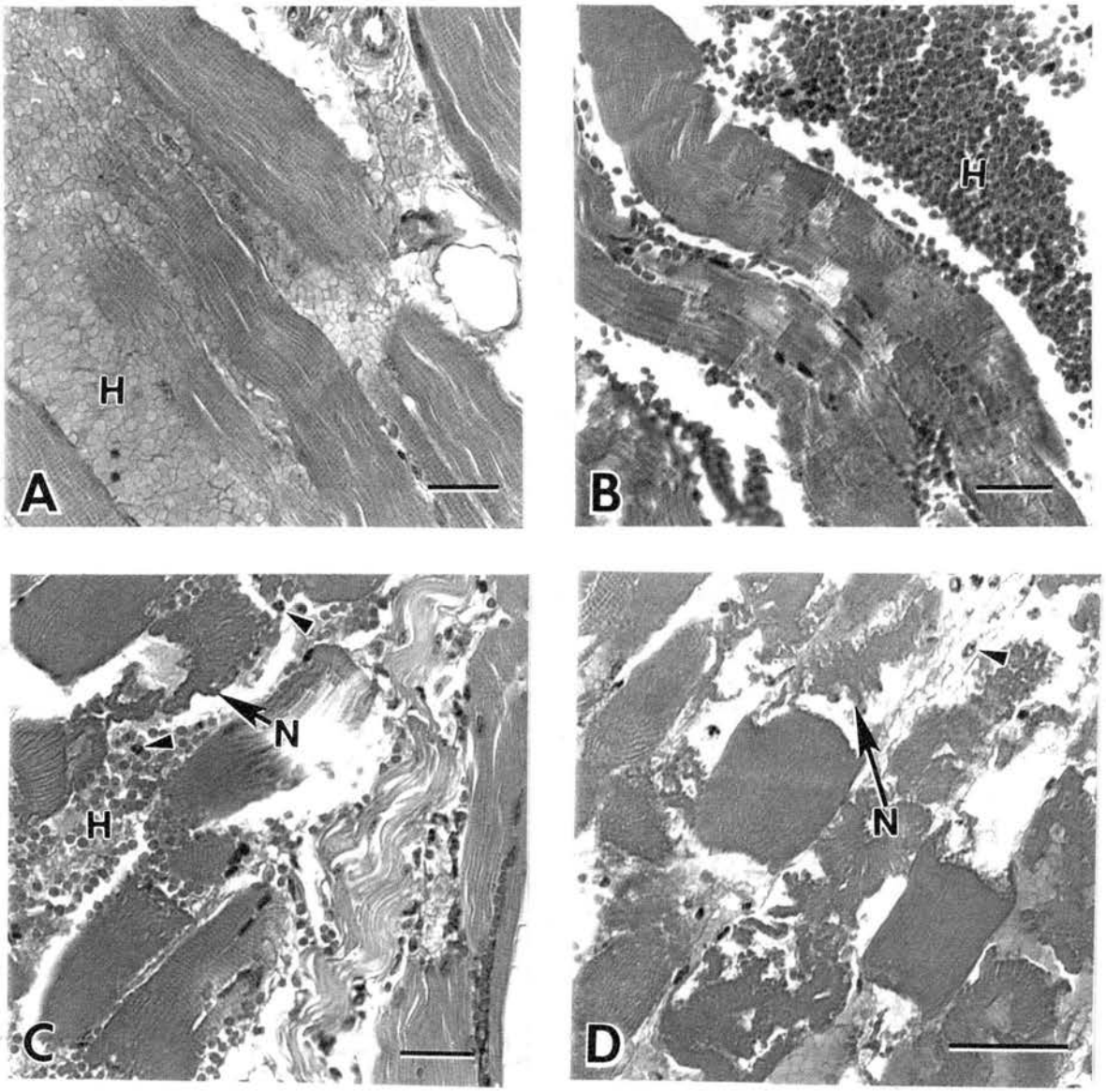
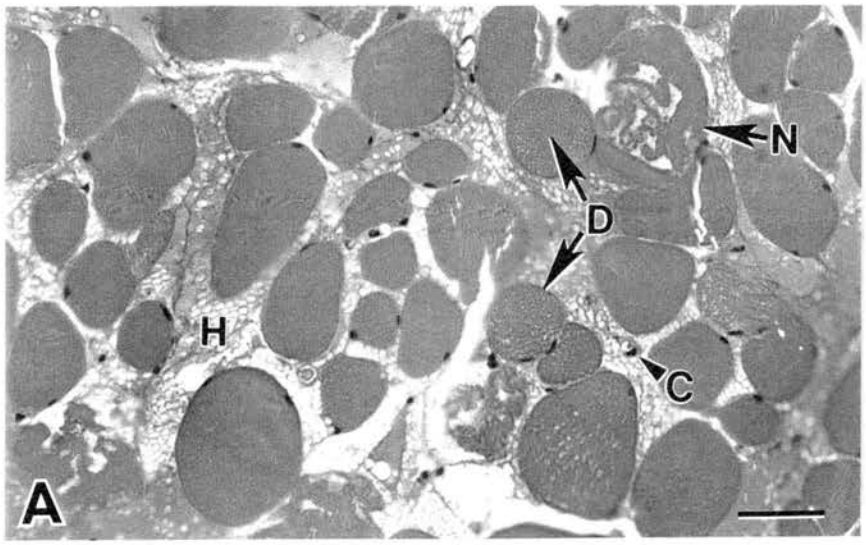

A
H
B
H
C
N
H
D
N

FIG. 3. LIGHT MICROGRAPHS OF SKELETAL MUSCLE AT 1 AND 24 HOURS AFTER INJECTION OF 5 μg/g PROTEINASE H. The micrographs show paraffin-embedded tissue stained with H & E. (A) Vacuolated skeletal muscle cells, D, necrotic skeletal muscle cell, N, intact capillary, C, and areas of hemorrhage showing extravasated and hemolysed erythrocytes, H. (Bar = 50 μm.) (B) Necrotic skeletal muscle cell, N, neutrophils, arrowheads, and hemorrhage, H, are visible. (Bar = 50 μm.)


N
D
H
C
A

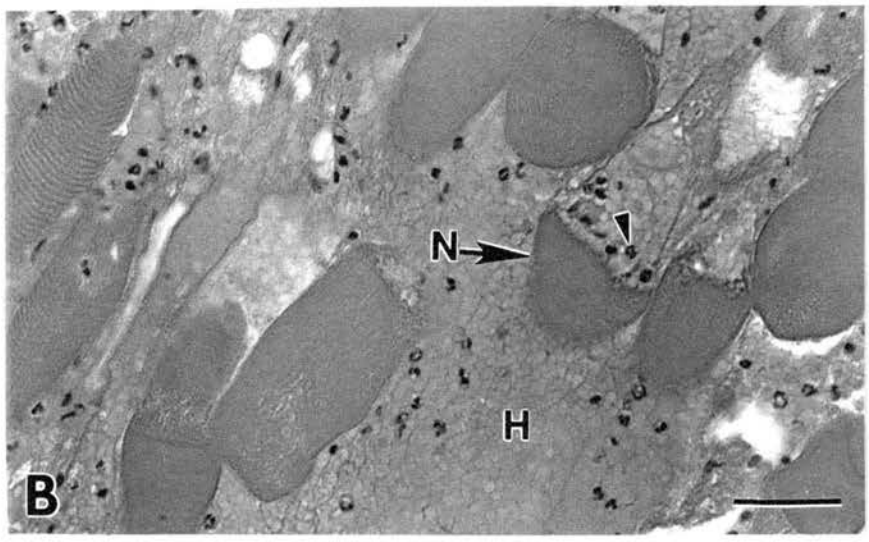
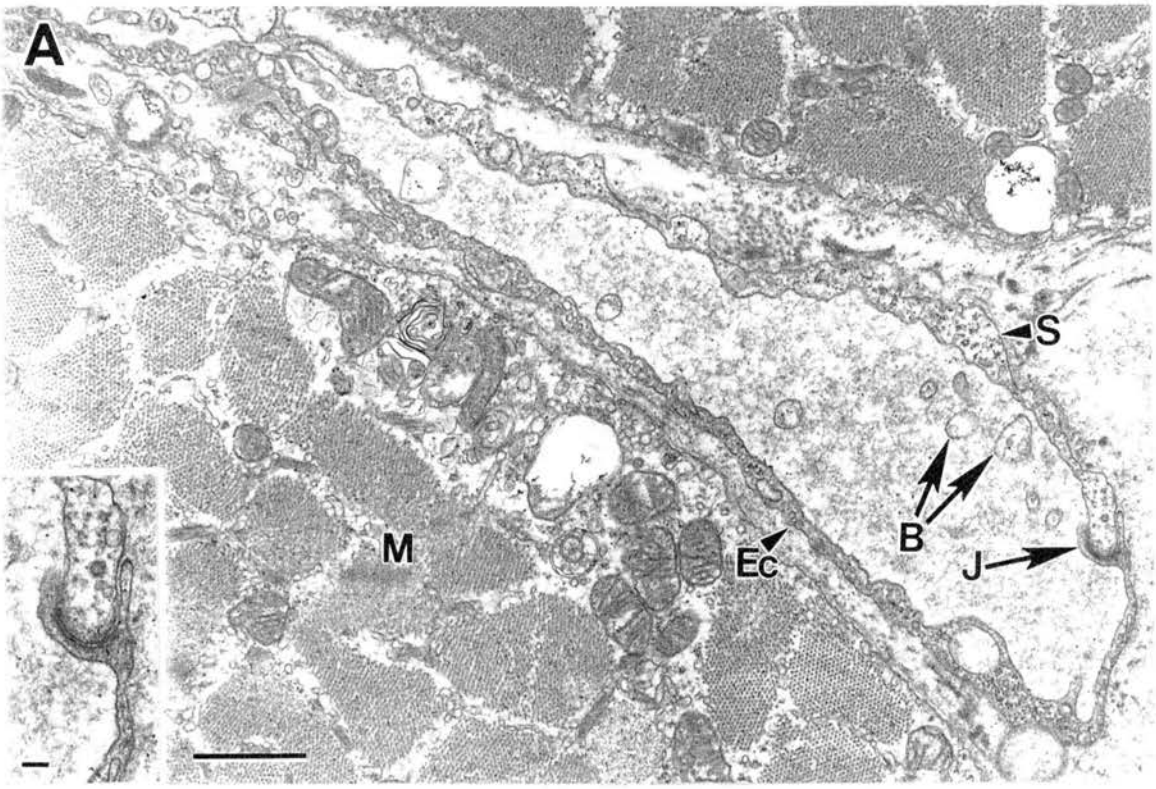

N
H
B

FIG. 4. ELECTRON MICROGRAPH OF DAMAGED CAPILLARY 10 MINUTES AFTER INJECTION OF PROTEINASE H. (A) A capillary with one swollen, S, and one non-swollen endothelial cell, Ec, with a swollen mitochondrion, Mi, is shown. Note the plasma membrane blebs, B, and intact intracellular junction, J, in the swollen endothelial cell. (Bar = 1 μm.)

(INSET) Magnified view of intact intracellular junction. (Bar = 0.1 μm.) (B) Higher magnification of a portion of the damaged endothelial cell pictured in Fig. 4A showing disruptions of the plasma membrane, arrowheads, and membrane blebs, B. (Bar = 0.2 μm.)


A
S
B
J
M
Ec

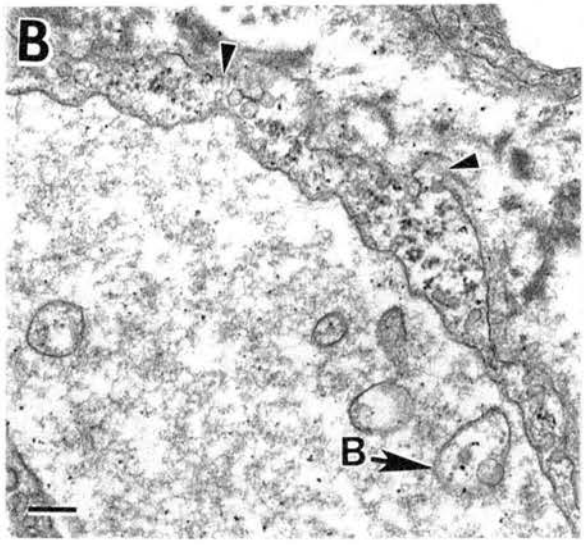

B
B

FIG. 5. ELECTRON MICROGRAPH OF RUPTURED CAPILLARY 10 MINUTES AFTER INJECTION OF PROTEINASE H. A large gap where the endothelial cell, Ec, is ruptured, double-headed arrow, membrane blebs, B, swollen mitochondrion, Mi, are shown. E, erythrocyte; M, skeletal muscle cell. (Bar = 1 μm.)

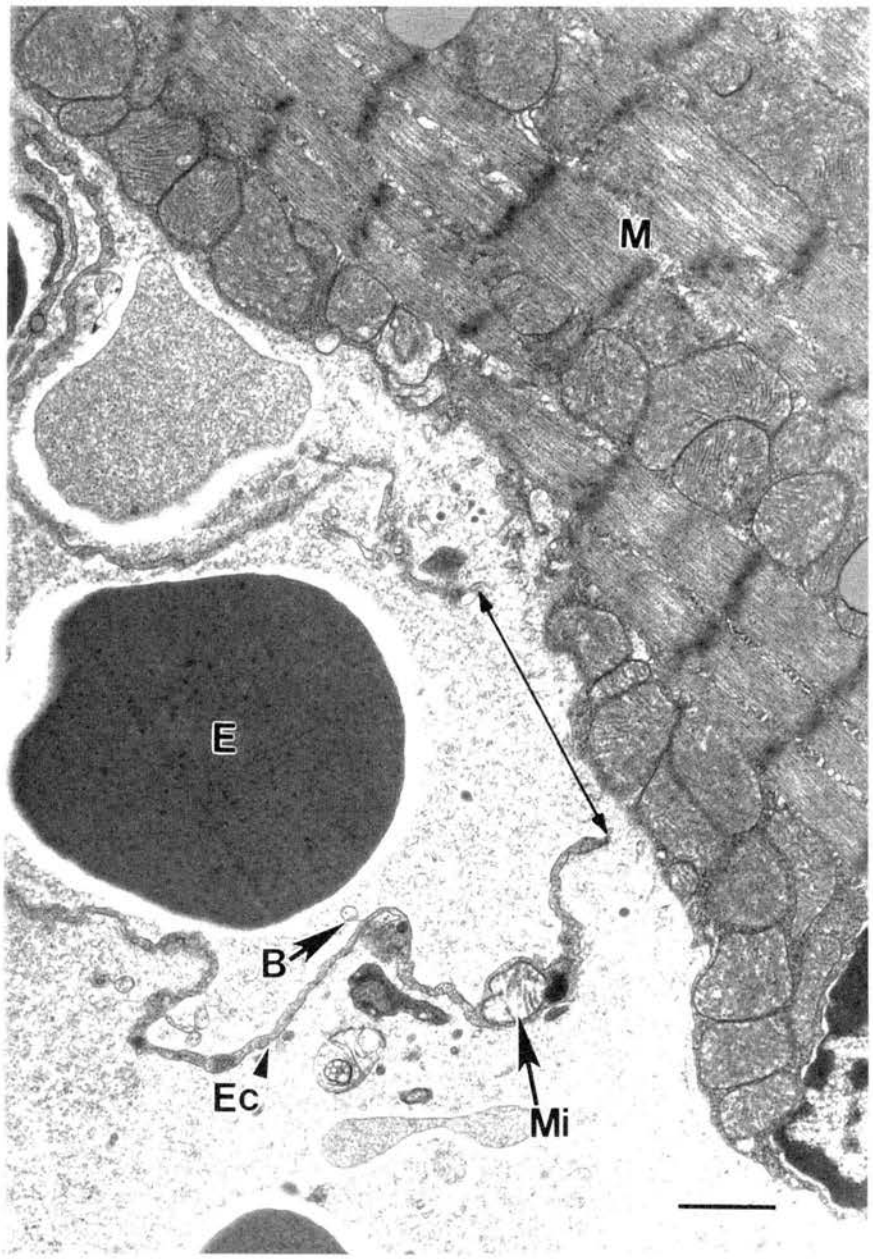
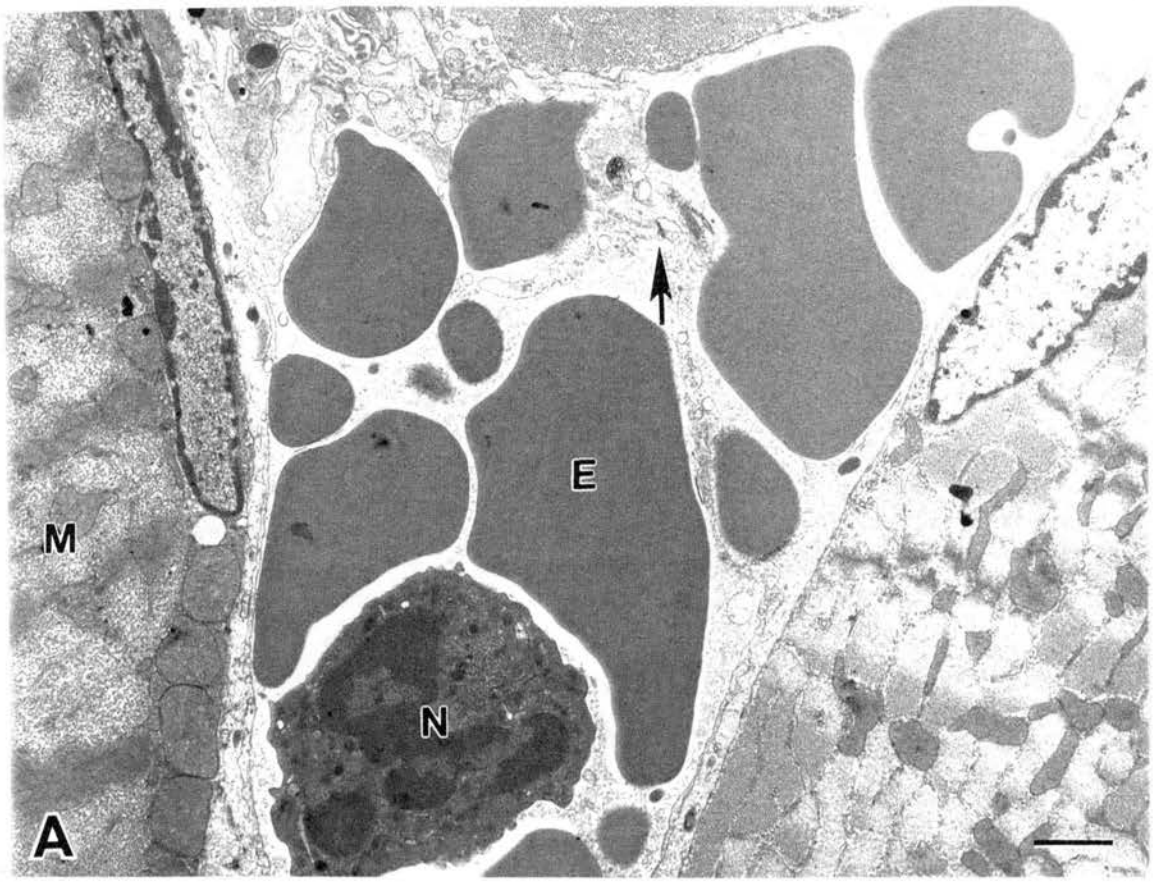
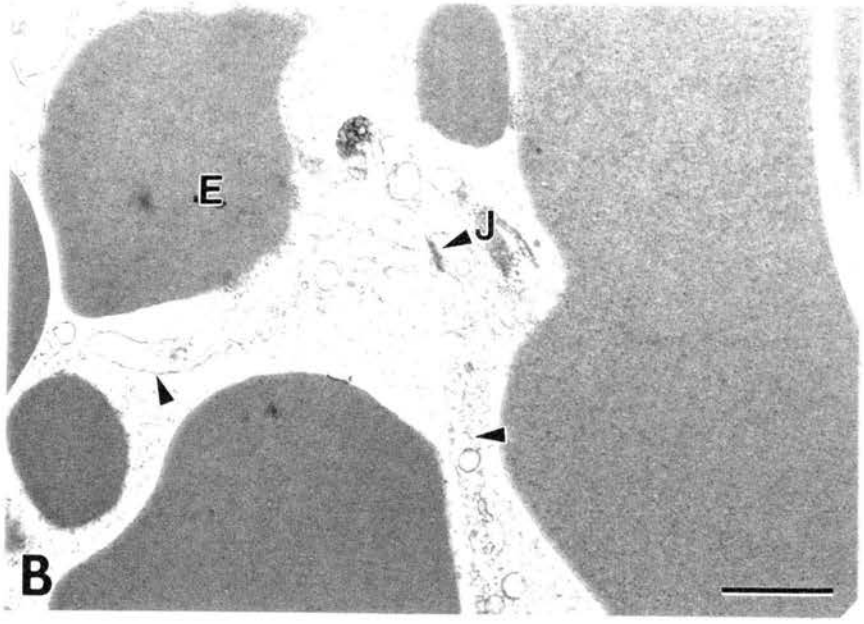

M
E
B
Ec
Mi

FIG. 6. ELECTRON MICROGRAPH OF RUPTURED CAPILLARY 10 MINUTES AFTER INJECTION OF PROTEINASE H. (A) M, skeletal muscle cell; N, neutrophil; E, erythrocyte. (Bar = 1 μm.) (B) Higher magnification of the area surrounding the arrow in Fig. 6A. J, intact intracellular junction; E, erythrocyte; arrowheads, endothelial cell fragments. (Bar = 1 μm.)


M
E
N
A


E
J
B

swollen mitochondria. The inset in Fig. 4A shows a high magnification of the intact intracellular junction between the two endothelial cells. In Fig. 5 frank rupture of a capillary endothelial cell, a swollen endothelial cell mitochondrion and blebbing of the lumenal plasma membrane of the endothelial cell is pictured. Fig. 6A and 6B shows an electron micrograph of a ruptured capillary 10 min after proteinase H (5 μg/g) injection. Extravasated erythrocytes are clearly seen escaping past the remnants of a ruptured capillary endothelial cell and its intact intracellular junction, shown at higher magnification in Fig. 6B. Cell swelling, mitochondrial swelling, blebbing, and rupturing of the plasma membrane (Figs. 4, 5, and 6) were some of the first indications of proteinase H-induced damage to capillary endothelial cells. Intracellular junctions were not visibly affected (Figs. 4A and B; Fig. 6B). These observations indicate that proteinase H-induces hemorrhage *per rhexis*.

DISCUSSION

Crude *C. adamanteus* venom has a very strong capacity to induce hemorrhage which is almost entirely attributed to the presence of a single hemorrhagic metalloprotease, proteinase H (KURECKI and KRESS, 1985).In general, crude snake venoms, especially those from the pit vipers, are known to induce vascular alterations and abnormalities in hemostasis (KORNALIK, 1991), endothelial cell integrity (OWNBY, 1982; LOMONTE *et al.*, 1994; BORKOW *et al.*, 1995), and blood flow which together aid in bringing about the demise of prey animals. Disruption of the blood supply to tissues is also debilitating and may aid in the immobilization of prey and in the degradation of body tissues to aid in digestion. Among the primary agents responsible for these disruptions are the venom hemorrhagic toxins. Most known snake venom hemorrhagic toxins cause

hemorrhage *per rhexis* (OWNBY, 1982; OWNBY, 1990). The mechanism of hemorrhage thus induced is thought to be the result of digestion of the endothelial basal lamina. Recent studies on the effects of hemorrhagic toxins on isolated endothelial cells *in vitro* demonstrate that the toxins tested caused endothelial cells to detach from the substratum but that they were not directly toxic to endothelial cells (MOREIRA *et al.*, 1994; BORKOW *et al.*, 1995). However, other studies on the pathogenesis of hemorrhagic toxins have shown that severe damage to endothelial cells might occur concomitantly or even prior to disruption of the basal lamina (OWNBY and GEREN, 1987). These findings are supported by data presented for hemorrhagic toxins from *Crotalus ruber ruber* and *Crotalus atrox* venoms indicating that these toxins are directly cytotoxic to cultured human umbilical vein endothelial cells (HUVEC) (OBRIG *et al.*, 1993). Certain venoms, however, have been reported to cause hemorrhage *per diapedesis*. These include venoms from *Echis coloratus* (SANDBANK *et al.*, 1974) and *Trimeresurus flavoviridis* (OHSAKA *et al.*, 1975). Thus it is becoming increasingly clear that different hemorrhagic toxins may operate by different mechanisms of action. The end result of envenomation is usually hemorrhage *per rhexis* regardless of the actual mechanism responsible for the hemorrhage.

Certain other events in the induction of hemorrhage *in vivo* suggest that the endothelium is directly damaged as a result of interaction with hemorrhagic toxins. The occurrence of platelet thrombi and inflammatory cells are suggestive of direct damage to the endothelium. Damage to endothelial cells results in the release of arachidonic acid metabolites, such as TxA_2, TxB_2, PGI_2, and LTB_4, PAF, cytokines (IL-1, IL-6, IL-8, TNF-α), and increased surface expression of adhesion molecules (ICAM-1, ICAM-2, ELAM) (JONES *et al.*, 1992; GERRITSEN and BLOOR, 1993; GRACE, 1994). PGI_2 is a powerful vasodilator to which vascular congestion might be attributed (MONCADA *et al.*, 1976). TxA_2

stimulates platelet aggregation (VANE *et al.*, 1990). Cytokines, LTB_4, and PAF (KUBES, *et al.*, 1990) stimulate neutrophil chemotaxis to the site of the injury.

The damage to muscle cells may be attributed to either ischemia or the action of PMNs associated with the inflammation observed. The events observed seem to indicate that both ischemia and inflammation had a role in inducing muscle cell damage. Myofibrillar clumping, mitochondrial damage, and the appearance of electron dense granules, possibly composed of calcium, within the mitochondria are other signs of necrosis that occur along with the appearance of PMNs. The most likely explanation for these observations is that the severe hemorrhage induced by proteinase H leads to ischemic damage of muscle cells which are then further damaged as a result of the action of PMNs which arrive to "clean up" the damaged tissues. It is impossible to determine, strictly on the basis of the data obtained in the current study, the exact primary cause of the considerable muscle cell damage that was observed. Either ischemia or inflammation, or both, may be involved in the muscle cell damage observed. The fact that the onset of muscle cell damage occurs at a relatively long time after the administration of proteinase H does suggest that this damage is probably secondary to ischemia and inflammation and not a primary effect of proteinase H. However, in one study (OWNBY *et al.*, 1978) hemorrhagic toxin *b* (HTb) from *C. atrox* venom appeared to induce myonecrosis directly.

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CHAPTER V

SYSTEMIC HEMORRHAGE INDUCED BY PROTEINASE H FROM *CROTALUS ADAMANTEUS* (EASTERN DIAMONDBACK RATTLESNAKE) VENOM

ABSTRACT

The systemic effects of a purified hemorrhagic toxin, proteinase H from *Crotalus adamanteus* venom, were studied. Female, white CD-1 mice were injected intravenously with proteinase H and tissue samples were obtained at 1, 3 and 24 hours after injection. Hemorrhage was observed grossly within 1 hour in several internal organs including the stomach and small intestine, the heart, and the lungs. Surface discolorations thought to be petechial hemorrhages were observed in the kidneys. The livers of treated animals were visibly swollen and darkened and lobules were accentuated. Tissue samples were taken from the stomach, duodenum, heart, lungs, liver, and kidneys and prepared for observation by light and electron microscopy. Frank hemorrhage was observed by light microscopy in the walls of the stomach and duodenum, in the myocardium, and in the lungs. Pulmonary hemorrhage was severe, with involvement of nearly all of the pulmonary tissue within 3 hrs. At doses of 5 μg/g, hepatic

degeneration was observed by 3 hrs. Renal glomeruli were noticeably swollen and the lumena of the proximal convoluted tubules indistinct. Closer examination by electron microscopy revealed that the endothelial cells comprising the fenestrated glomerular capillaries remained intact but signs of degeneration (i. e., cytoplasmic swelling, mitochondrial swelling) were observed. Proteinase H induces systemic hemorrhage in the heart, lungs, stomach, and small intestine, renal glomerulonephropathy, and hepatic degeneration.

INTRODUCTION

Several snake venoms and snake venom hemorrhagic toxins have long been known to cause systemic hemorrhage. Systemic hemorrhage has been induced in several organs including the lungs, brain, heart, kidneys, gastrointestinal mucosa, pancreas, liver, adrenal glands, and thyroid by the experimental administration of snake venom (PEARCE, 1909; TAUBE and ESSEX, 1937; FIDLER *et al.*, 1940; NIKAI, *et al.*, 1985; NIKAI, *et al.*, 1986). A few snake venom hemorrhagic toxins have also been shown to cause systemic hemorrhage (NIKAI *et al.*, 1985; NIKAI *et al.*, 1986; KAMIGUTI *et al.*, 1992) and thereby contribute to the lethality of snake venoms.

Proteinase H is a highly hemorrhagic metalloprotease isolated from *C. adamanteus* venom. The entire hemorrhagic capacity of this venom has been shown to be due to proteinase H (KURECKI and KRESS, 1985). In the current study the ability of proteinase H to induce systemic hemorrhage in mice was investigated by light and electron microscopy.

MATERIALS AND METHODS

Purification of proteinase H

Crude *Crotalus adamanteus* venom (lot No. CA/87A) was obtained in lyophilized form from Biotoxins, Inc. (St. Cloud, FL, U. S. A.) and stored desiccated at -20°C until use. The venom was fractionated according to the method described by KURECKI and KRESS (1985), with minor modifications (Chapter II). In the last step of the fractionation a Waters DEAE-5PW semi-preparative HPLC anion exchange column was used in lieu of the open column DEAE-Sepharose used by Kurecki and Kress. Homogeneity of the purified toxin was demonstrated by the formation of a single band on a SDS-PAGE gel stained with silver nitrate and by analytical C18 reversed-phase HPLC.

Sample preparation and microscopy

Experimental female mice (CD-1, Charles River) weighing 22-24 g were injected with 100 µl of a physiological saline solution (PSS) containing a final dose of either 1 or 5 µg toxin/g mouse weight. Control mice were injected with 100 µl of PSS. All mice were injected intravenously via the median tail vein and were killed by cervical dislocation at either 1, 3, or 24 hours following injection. Tissue samples were taken from the following organs: heart, lungs, kidney, liver, stomach, duodenum. All 6 of these organs were taken from every mouse treated. A total of twenty-one mice, three per treatment and three control mice, were used in two separate experiments (n=3).

After sampling, the tissue was immediately processed for embedding in either paraffin or plastic resin. For embedding in paraffin tissue samples were fixed in Bouin's solution overnight and then washed overnight in three changes of 70 % ethanol. The tissue thus fixed was then embedded in paraffin and 3 µm

sections were taken, mounted with Permount on glass slides, and stained with hematoxylin and eosin for viewing. Tissue samples were embedded in plastic resin as previously described by OWNBY and GEREN (1987) by initial fixation in 2 % glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) followed by embedding in Polybed resin (Polysciences, Warrington, PA, U. S. A.). Thick (0.7 µm) sections were taken from blocks of the plastic-embedded tissue using a Sorvall MT-5000 ultramicrotome with glass knives and were stained with Mallory's trichrome for light microscopy. Blocks were randomly selected for sectioning (n=5).

Thin sections (70-90 nm) of selected areas of the plastic embedded blocks were taken for electron microscopy with a Sorvall MT-6000 ultramicrotome using a diamond knife. Sections were picked up on copper grids (200 mesh) and stained with methanolic uranyl acetate and lead citrate, observed and photographed with a JEOL 100 CX TEMSCAN electron microscope at an accelerating voltage of 80 KV.

RESULTS

GROSS OBSERVATIONS

Following injection of the low dose of proteinase H (1 µg/g) mice were not noticeably distressed or otherwise adversely affected. However, mice injected with the higher dose of proteinase H (5 µg/g) were noticeably affected within 5 minutes after injection: piloerection was observed over the entire body, mice were lethargic and respiration was slowed. These mice died within 3 hrs after injection.

Heart

Upon dissection the hearts of mice injected with proteinase H (5 μg/g) were darkened and surface petechiae were visible. When the heart itself was dissected areas of hemorrhage were visible within the cut surface of the myocardium.

Lungs

Upon gross observation petechiae were observed on the lungs of mice injected with proteinase H (1 μg/g) at 3 and 24 hrs after injection. The intensity and extent of petechiation increased in a time dependent manner. In mice injected with a higher dose of proteinase H (5 μg/g) frank pulmonary hemorrhage involving nearly all of both lungs was observed 3 hrs after injection.

Liver

Livers from mice injected with low (1 μg/g) doses of proteinase H were not visibly affected by the toxin upon gross examination at all time periods through 24 hrs. Livers from mice injected with 5 μg/g of proteinase H were noticeably wet and heavy 1 hr after injection. In the latter mice, at 3 hrs after injection, an enhanced centrilobular pattern was observed on the external and cut surfaces of the liver.

Stomach

The stomachs of mice in the low-dose group had a normal, healthy appearance at all time periods. In the high-dose group petechial hemorrhages were observed at 1 hr on the serosal surface of the corpus and pylorus of the stomach; the mucosal surface of the stomach appeared normal. In these mice, at 3 hrs, large ecchymoses were observed on the serosal and mucosal surfaces of the corpus and pylorus of the stomach.

Small intestine (Duodenum)

The small intestines of mice in the low-dose group were not noticeably affected. In the large-dose group petechiae and ecchymoses were observed on the serosal surface of the small intestine at 1 hr In these mice, at 3 hrs, hemorrhage was observed throughout the entire serosal surface of the small intestine.

Kidneys

The kidneys of mice in the low-dose group had a normal appearance. A diffuse petechiation was observed on the surface of kidneys from mice in the high-dose group at 3 hrs after injection. Petechiation was also observed in the cut-surface of the renal cortex of kidneys from these same mice.

HISTOLOGY

Heart

Interstitial hemorrhage throughout the myocardium was observed by light microscopy and cross-striations in cardiac myocytes were disrupted (Fig. 1B) compared to control (Fig. 1A). Vessels larger than capillaries remained

intact.

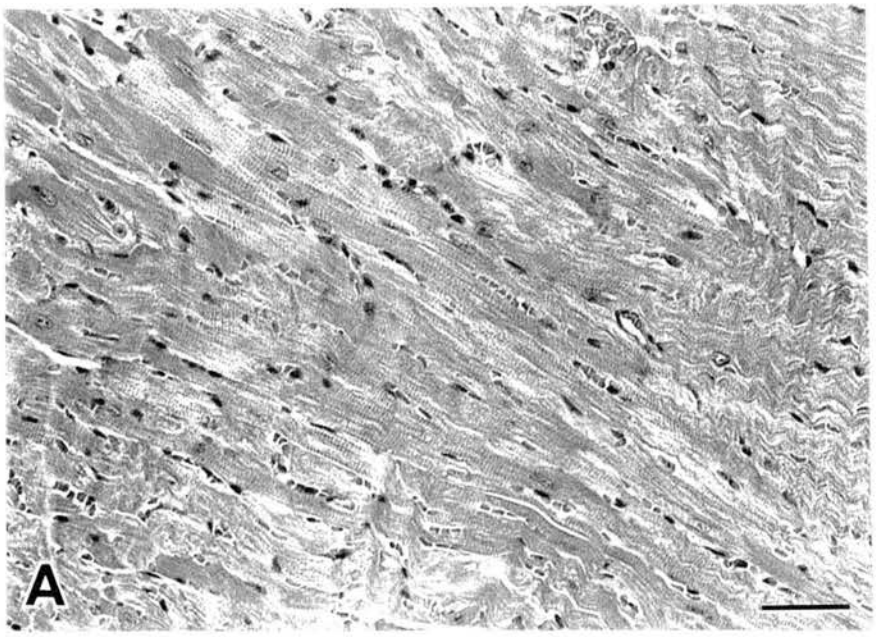
Lungs

Histological observations of lungs from mice treated with low doses of proteinase H (1 μg/g) compared with normal control lungs (Fig. 2A) revealed that alveolar hemorrhage was time dependent, beginning as small foci of hemorrhage which spread throughout the tissue (Fig. 2B). In mice treated with 5 μg/g proteinase H intense and diffuse hemorrhage was observed within 1 hr. By 3 hrs the normal architecture of the respiratory tissue was completely disrupted and no normal tissue is visible (Fig. 2C).

Liver

In the low-dose group, light microscopy comparison of normal livers (Fig. 3A) with livers from proteinase H-treated mice revealed glycogen-depletion of hepatocytes radiating outward from the central vein at 1 hr and 3 hrs after injection. Glycogen was consistently leached from hepatocytes in control groups making the cytoplasm stain lightly and in an irregular pattern. Glycogen-depletion was indicated by the homogeneous acidophilic staining of hepatocyte cytoplasm. By 24 hrs after injection the histological appearance of the liver was normal. In the high-dose group at 1 hr after injection the pattern of glycogen-depletion was again apparent and by 3 hrs hepatocytes showed obvious signs of degeneration including swelling, vacuolation, and nuclear pyknosis (Fig. 3B).

FIG. 1. LIGHT MICROGRAPHS OF CARDIAC MUSCLE. (A) Control tissue following i. v. injection of PSS. (Bar = 50 μm.) (B) Cardiac muscle 3 hours after i. v. injection of proteinase H (5 μg/g). Note extensive hemorrhage, H, throughout the interstitial spaces of the myocardium. (Bar = 50 μm.)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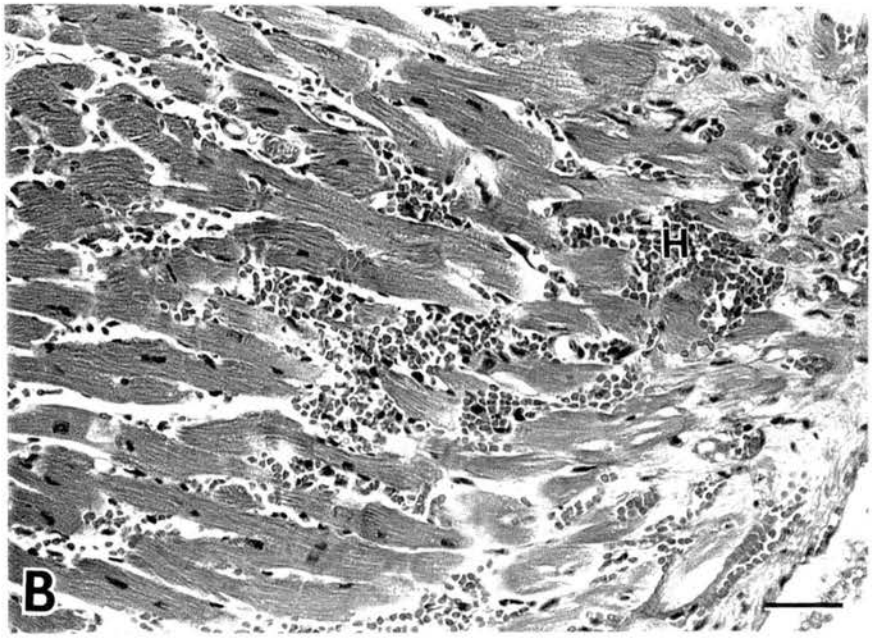
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FIG. 2. LIGHT MICROGRAPHS OF LUNG TISSUE. (A) Control lung after i. v. injection of PSS. A, alveolar space; arrowhead, alveolar capillary. (Bar = 50 μm.) (B) 24 hours after i. v. injection of proteinase H (1μg/g). H, hemorrhage; A, alveolar space; arrowhead, alveolar capillary. (Bar = 50 μm.) (C) 3 hours after i. v. injection of proteinase H (5 μg/g). Note extensive hemorrhage, H, and loss of normal histological architecture. (Bar = 50 μm.)

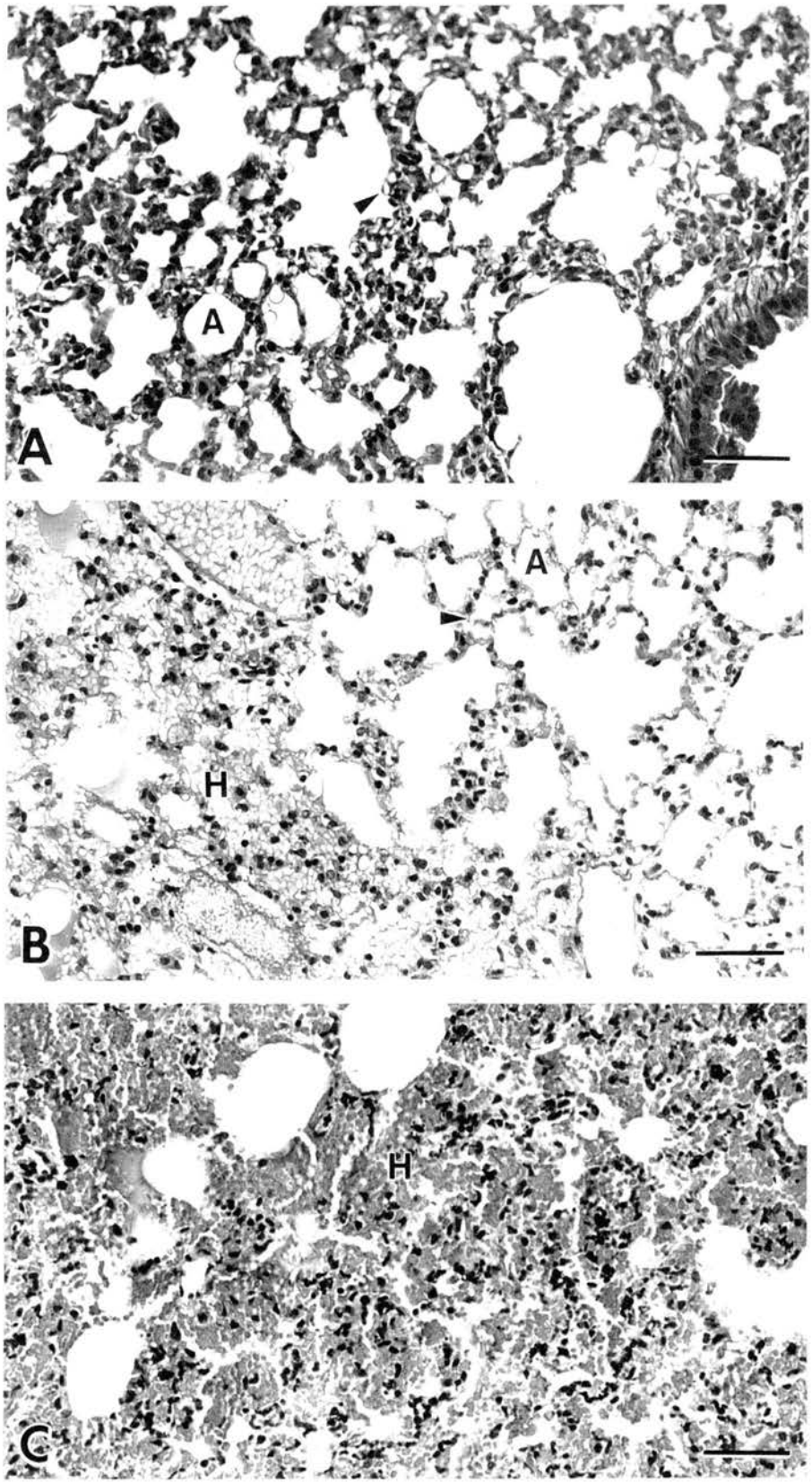
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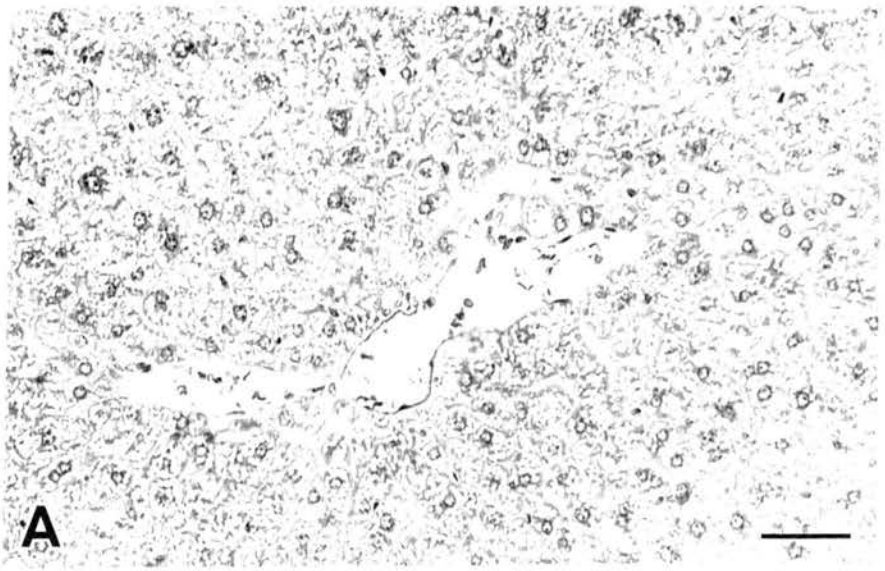
FIG. 3. LIGHT MICROGRAPHS OF LIVER. (A) Control liver 3 hours after i. v. injection of PSS. (Bar = 50 μm.) (B) Liver 3 hours after i. v. injection of proteinase H (5 μg/g). Nh, vacuolated, degenerative hepatocyte with pyknotic nucleus. (Bar = 50 μm.)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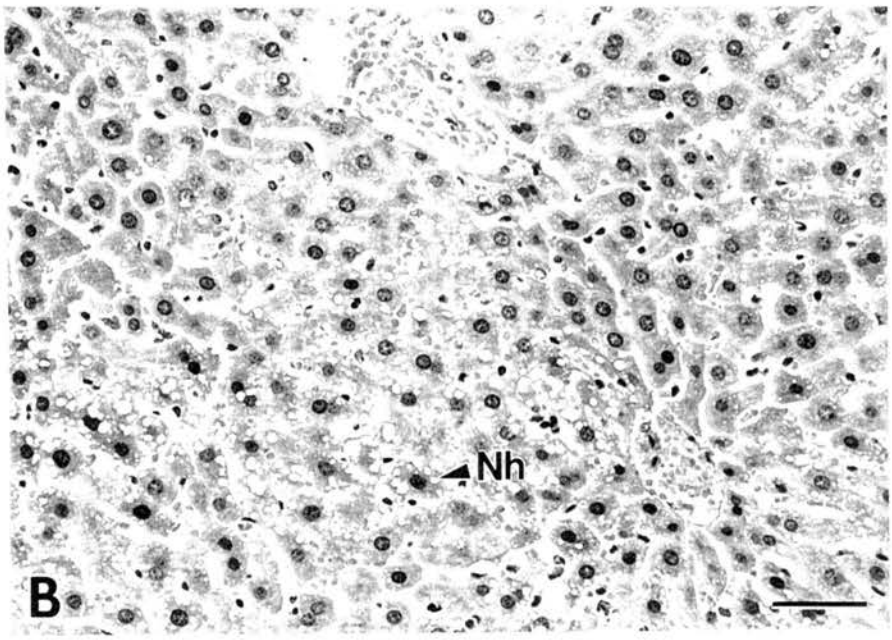
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Stomach

By comparison with normal murine stomachs (Fig. 4A) hemorrhage was observed throughout the lamina propria of the tunica mucosa (Fig. 4B) and in the tunica adventitia of the stomach.

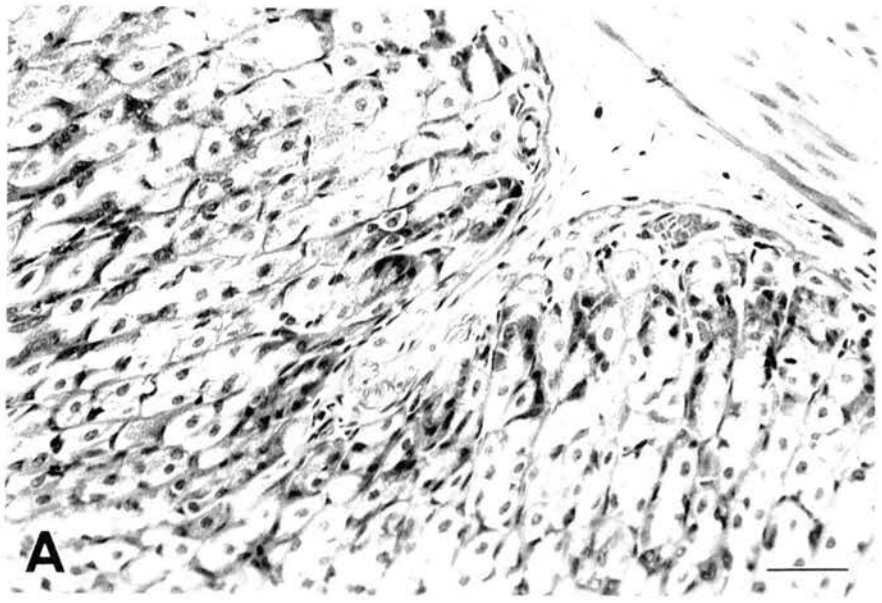
Duodenum

At 3 hrs after injection of proteinase H (5 μg/g) extensive hemorrhage was observed throughout the lamina propria, muscularis mucosa, submucosa and tunica adventitia of the duodenum (Fig. 5B) in comparison with control duodenum (Fig. 5A).

Kidneys

Light microscopic observation of paraffin and thick plastic sections revealed proximal convoluted tubule with indistinct lumens and swelling and congestion of the glomerular capillaries in the high-dose group 3 hrs after injection as compared to control kidneys (Fig. 6A). The glomerular architecture was intact (Fig. 6B), but swelling decreased the size of the Bowman's space. At the electron microscopic level capillary fenestrae were obscured, capillaries were congested with erythrocytes and endothelial cells showed signs of degeneration including swelling of the cell and mitochondria and dilatation of the endoplasmic reticulum (Fig. 7B). There was no apparent change in the appearance of the basal laminae of the glomerular capillaries. A glomerulus from a control kidney is pictured in Fig. 7A.

FIG. 4. LIGHT MICROGRAPHS OF PYLORIC STOMACH. (A) Control 3 hours after i. v. injection of PSS. (Bar = 50 μm.) (B) Pyloric stomach 3 hours after i. v. injection of proteinase H (5 μg/g). Note areas of hemorrhage, H, containing extravasated erythrocytes, arrowheads, throughout the lamina propria. (Bar = 50 μm.)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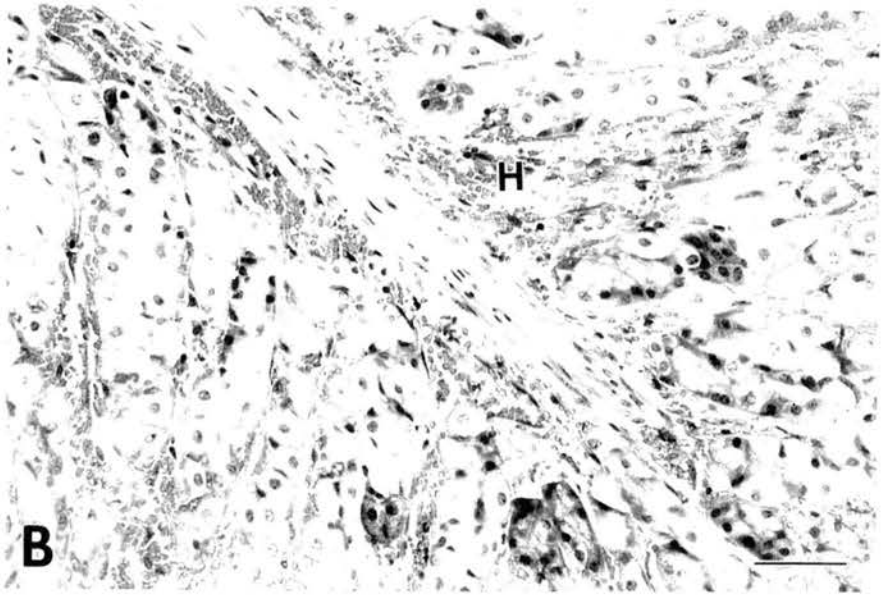
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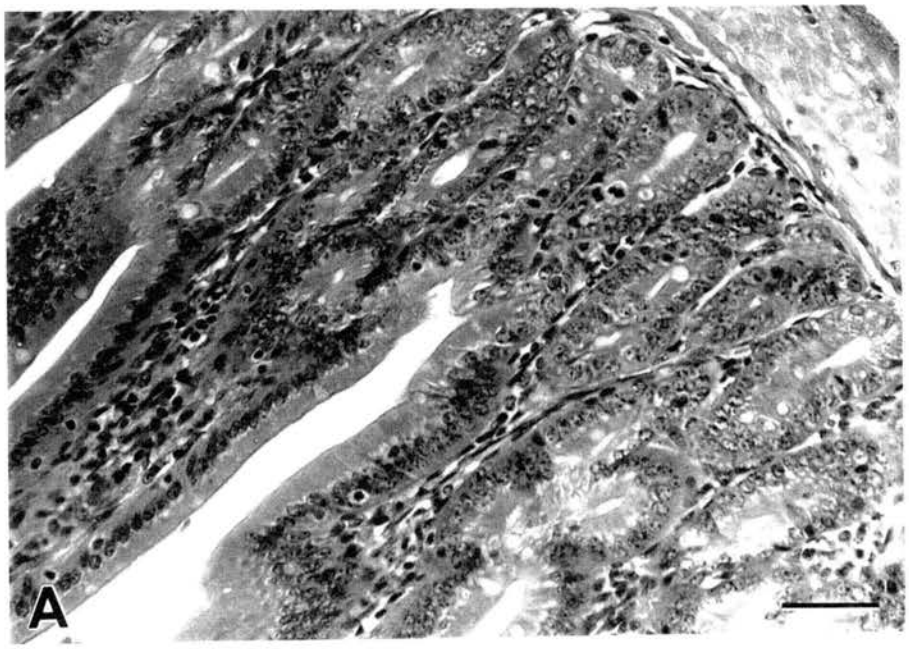
FIG. 5. LIGHT MICROGRAPHS OF DUODENUM. (A) Control 3 hours after i. v. injection of PSS. (Bar = 50 μm.) (B) Duodenum 3 hours after i. v. injection of 5 μg proteinase H/g. Note area of extensive hemorrhage, H, containing extravasated erythrocytes, arrowheads, in the submucosa, muscularis mucosa and lamina propria; arrowhead, longitudinal view of capillary. (Bar = 50 μm.)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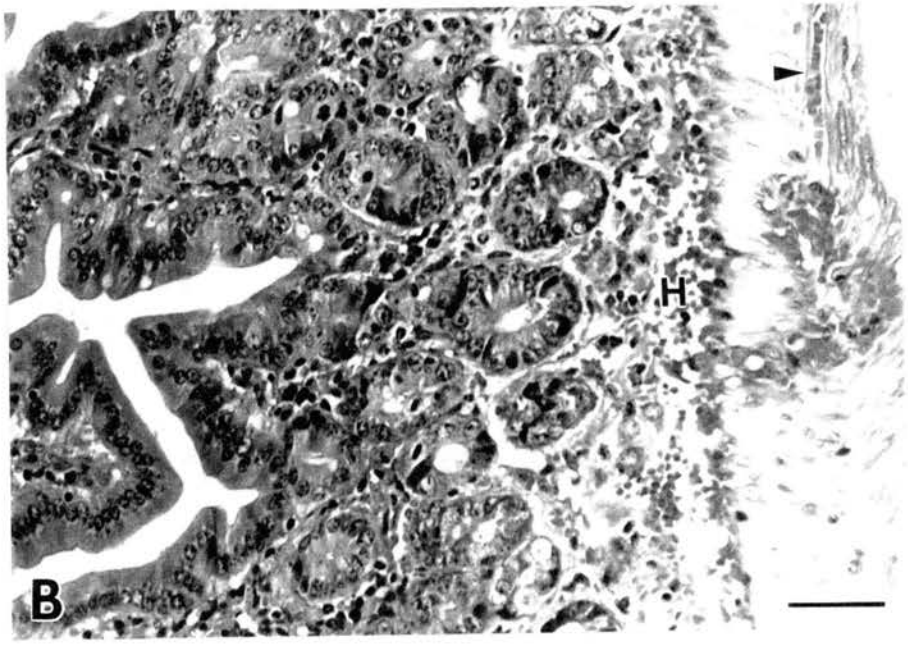
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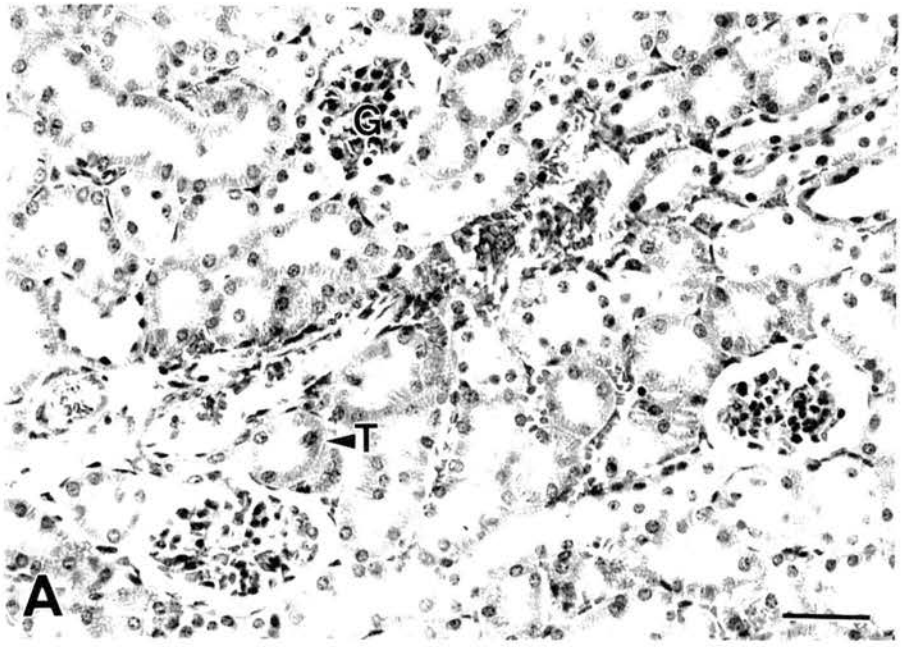
FIG. 6. LIGHT MICROGRAPHS OF RENAL CORTEX. (A) Control kidney 3 hours after i. v. injection of PSS. G, glomerulus. (Bar = 50 μm.) (B) Kidney 3 hours after i. v. injection of proteinase H (5 μg/g). G, glomerulus; arrowhead, swollen glomerular capillary congested with erythrocytes. (Bar = 50 μm.)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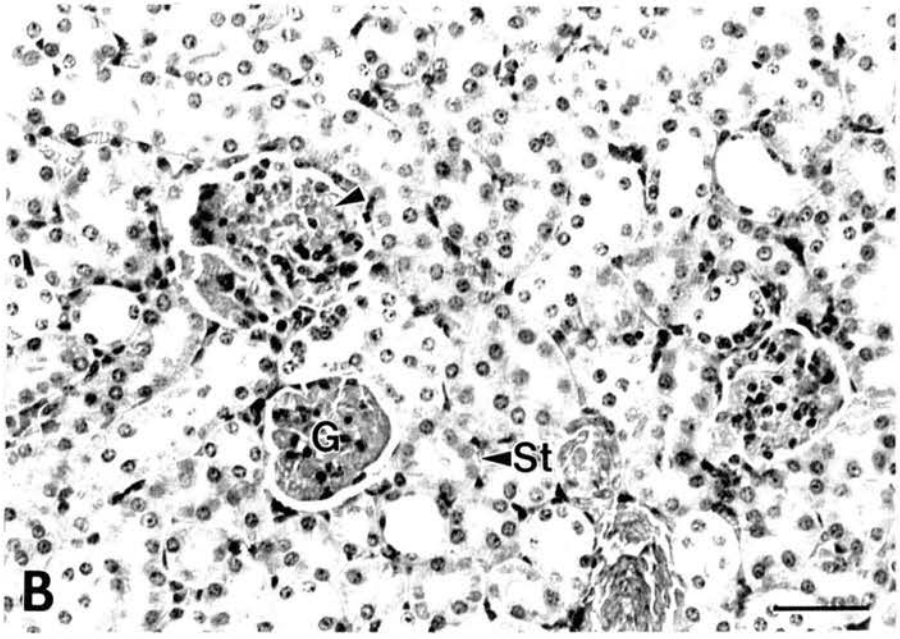
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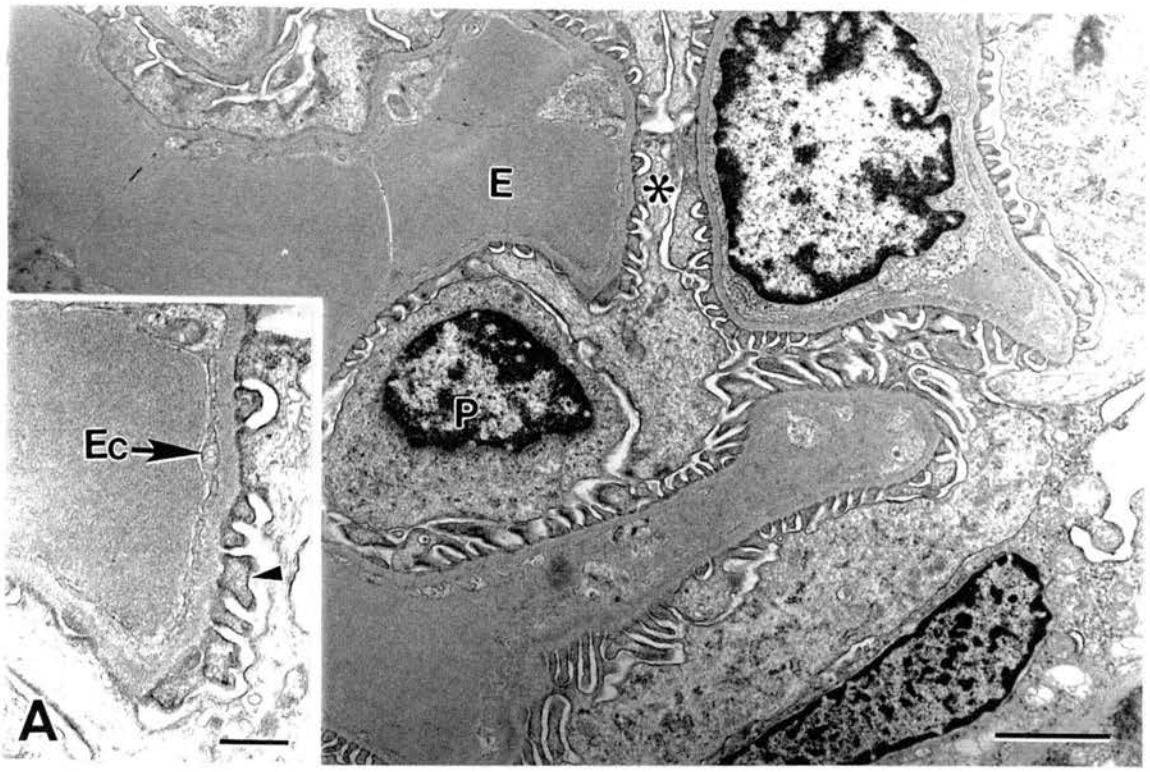
FIG. 7. ELECTRON MICROGRAPHS OF GLOMERULI OF THE KIDNEY. (A) Control glomerulus 3 hours after i. v. injection of PSS. E, erythrocyte; P, podocyte. (Bar = 1 μm.) (INSET) Higher magnification of area surrounding asterisk showing a portion of the endothelial cell, Ec, of the fenestrated glomerular capillary and podocyte pedicel, arrowhead. (Bar = 0.5 μm.) (B) Renal glomerulus 3 hours after i. v. injection of 5 μg proteinase H/g showing swollen and congested glomerular capillary. E, erythrocyte; arrowhead, podocyte pedicel. (Bar = 2 μm.) (INSET) Higher magnification of glomerular capillary adjacent to asterisk showing cytoplasmic and mitochondrial swelling and dilatation of the endoplasmic reticulum of the endothelium of a glomerular fenestrated capillary. Ec, endothelial cell; Mi, swollen endothelial mitochondrion; Er, dilatated endoplasmic reticulum; arrowhead, podocyte pedicel.
(Bar = 0.5 μm.)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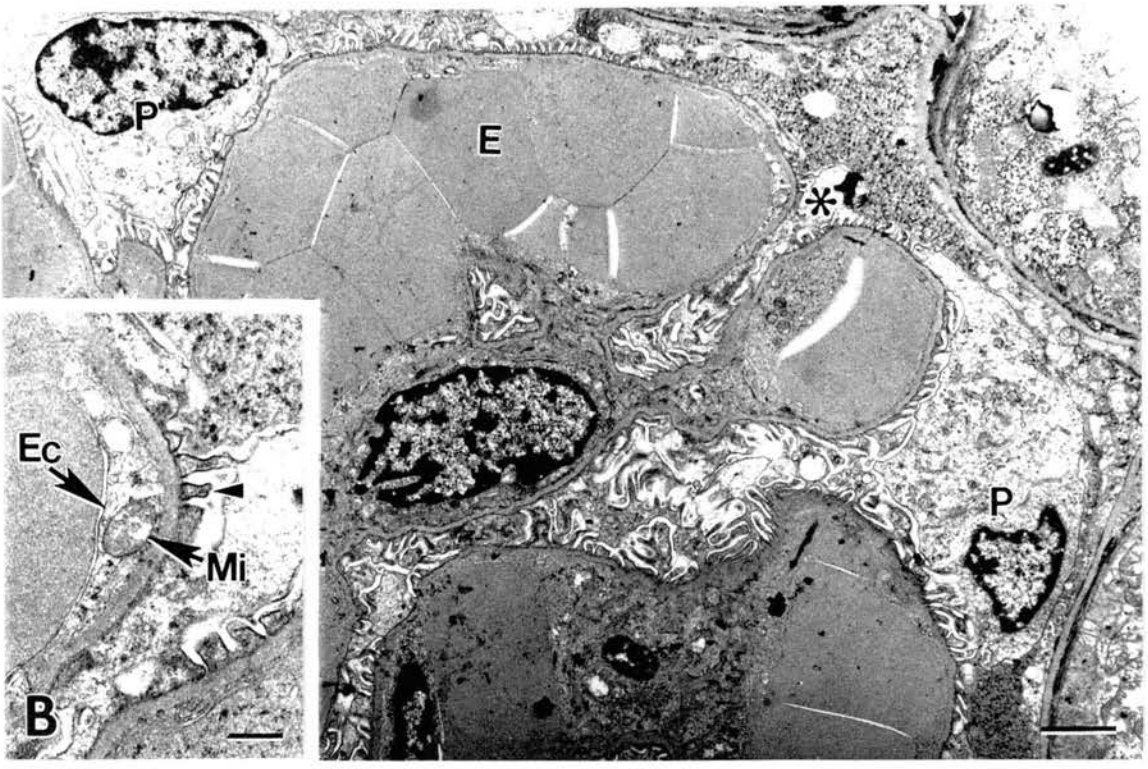
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DISCUSSION

Hemorrhage is generally a complication of snake bite envenomation that affects the local tissues (OWNBY, 1990). However, in several viperine venoms, particularly *Bothrops* venoms, systemic hemorrhage is the principal cause of death, especially in human victims (KAMIGUTI *et al.*, 1992). In many cases it is thought that systemic hemorrhage is the result of snake venom components, other than hemorrhagic metalloproteinases, that induce coagulopathies (KORNALIK, 1991), although hemorrhagic toxins have been shown to contribute to systemic hemorrhage (OHSAKA, 1979). Few hemorrhagic toxins have been shown to definitively cause systemic hemorrhage by themselves (NIKAI *et al.*, 1985; NIKAI *et al.*, 1986).

The present study conclusively demonstrates that a single hemorrhagic toxin, proteinase H, is able to induce hemorrhage in a number of internal organs (i. e., heart, lungs, stomach, duodenum) and other pathological changes in the liver and kidneys. Hemorrhage is presumably caused *per rhexis* as proteinase H and many other similar toxins have been shown to cause hemorrhage by this mechanism (Chapter IV; OWNBY, 1982) When administered intravenously to mice at high doses (5 μg/g), proteinase H was able to cause death within 3 hrs. Death was presumably the result of respiratory failure due to the destruction of the respiratory epithelium as a result of severe pulmonary hemorrhage. Severe and extensive hemorrhage in the heart and gastrointestinal tract (stomach and small intestine) undoubtedly contributed to the lethality of the toxin. Less severe hemorrhage was also induced in the lungs at a lower dose (1 μg/g) in less than 3 hrs. Mice treated with this low dose survived through 24 hrs.

Hemorrhage did not occur in the liver, although other pathological alterations were observed. The enhanced lobular pattern of the liver and the

vacuolar degeneration of hepatocytes was suggestive of hepatocellular degeneration (MACLACHLAN and CULLEN, 1988) possibly due to ischemic and anaerobic hypoxia (SLAUSON and COOPER, 1990). These effects were most likely brought about by decreased cardiac output and hypoxia resulting from the diminished oxygenating capacity of the lungs. Decreased cardiac output can be inferred from the obviously compromised pulmonary venous return and negative inotropy due to cardiac myocyte pathology induced by hypoxia in the heart. The pathological changes in the liver therefore cannot be conclusively attributed to the primary action of proteinase H and may in fact be a secondary result of the toxin's action, although a direct toxic effect cannot be ruled out

In the kidneys, the swelling of the tubular epithelium could be explained by hypoxic ischemia and the alterations in glomerular capillaries were indicative of damage to renal endothelium. The congestion and swelling of the glomerular capillaries was a paradoxical finding. Hypoxia and a decrease in circulating blood volume should have led to constriction of the afferent arterioles perfusing the glomeruli (WEST, 1990). Instead, the glomeruli were hyperemic and swollen. This may be explained by the damage to the glomerular endothelium. These findings differ from those from experimental envenomations with other Crotalid venoms (i. e., *C. atrox, C. durissus terrificus*) in which damage to the visceral epithelium, proximal convoluted tubules and the glomeruli occurred (TU, 1977)

Proteinase H affected only the capillaries of all the tissues sampled. This is consistent with the conclusions of MITCHELL and REICHERT in 1886 (Cited by OHSAKA, 1979) and LOMONTE *et al.* (1994) that snake venom induced hemorrhage occurred only via the capillaries and that arteries and veins were unaffected.

Interestingly, the effect of proteinase H was not as strong in the kidney or liver as it was in the other organs, particularly the lungs and heart. This is not

surprising as the toxin is exposed to these organs first due to the route of injection via the median tail vein. This is also consistent with the finding that ^{135}I-labeled *Agkistrodon piscivorus* venom is concentrated initially in the lungs (DEVI, 1971). These observations and the findings presented in the current study suggest that proteinase H has a selective effect on the endothelium of different tissues and internal organs.

The findings presented in this study also indicate that a hemorrhagic toxin can cause severe and extensive hemorrhage of capillaries in various tissues when presented to the lumenal surface of the endothelium, away from the basal lamina. This does not necessarily conflict with the idea that hemorrhagic toxins cause hemorrhage by degrading the basal lamina, but it does bring forth some interesting ideas. Proteinase H still may be causing hemorrhage by digesting the basal lamina but, except in fenestrated capillaries, it must cross the endothelial barrier first before this can occur. However, in the kidneys, proteinase H clearly caused damage to the glomerular endothelium without visibly affecting the basal lamina, even at the electron microscopic level. These glomerular lesions were very different to those reported by PEARCE (1909) following treatment of with crude *C. adamanteus* venom. PEARCE stated that *C. adamanteus* venom caused, "...a very striking and interesting exudative glomerular lesion without evident alteration of the tubular epithelium," and that this venom caused intraglomerular hemorrhage often with exudation and free hemorrhage into the Bowman's capsule. In the present study these latter changes were not observed.

In summary, we have shown that intravenous administration of proteinase H causes hemorrhage in many internal organs including the heart, lungs, stomach and small intestine. At high doses proteinase H also induces hepatocellular degeneration within 3 hrs of administration. In addition, proteinase H causes glomerulonephropathy in the form of injury to the

endothelial cells of the fenestrated capillaries of the glomeruli without apparent alteration to the endothelial basal lamina.

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CHAPTER VI

EFFECT OF PROTEINASE H, THE MAIN HEMORRHAGIC TOXIN OF *CROTALUS ADAMANTEUS* VENOM, ON ENDOTHELIAL CELLS *IN VITRO*

ABSTRACT

Human umbilical vein endothelial cells (HUVEC), human dermal microvascular cells (HMVEC), and rat aorta endothelial cells (RAEC) were used to study the *in vitro* effects of proteinase H on cultured endothelial cells. Proteinase H induced similar morphological changes in HUVEC and HMVEC, but not in RAEC. Early changes in these cells took the form of the appearance of spaces in the monolayer or retraction of cells from each other. The cells eventually rounded up and detached from the substratum. Proteinase H was preferentially cytotoxic to HMVEC as assessed by Trypan blue dye uptake, lactate dehydrogenase release and MTT viability assays. All of these effects were time and concentration dependent. Proteinase H was not cytotoxic to HUVEC nor to RAEC. A decreased plating efficiency of the detached HMVEC was further evidence that proteinase H was directly cytotoxic to HMVEC.

INTRODUCTION

Hemorrhage is one of the most problematic symptoms accompanying envenomation by crotalid snakes in the United States. In most cases this hemorrhage is apparent only as local tissue damage but in severe envenomations considerable systemic hemorrhage may occur in internal organs such as the kidneys, brain, lungs, heart, liver, and intestines (TU, 1977). As such, hemorrhage can often be the major contributing factor to the lethality of a venom (OHSAKA, 1979; MEBS, *et al.*, 1988). Many different hemorrhagic toxins have been isolated from snake venoms, and the hemorrhagic activity of a venom is generally attributed to the presence of such toxins.

The only consistent clinical treatment of snake bite in the United States is the administration of polyvalent (Crotalidae) antivenom produced by Wyeth-Ayerst (Marietta, PA, U. S. A.). Unfortunately this antivenom may not be particularly effective in reducing the hemorrhage that occurs in actual snake bites. One of the four venoms used in the production of this antivenom is that of *Crotalus adamanteus,* a highly hemorrhagic venom. Due to these two facts the venom is of particular interest in considering the ability of an antivenom to neutralize venom induced hemorrhage. A better understanding of the mechanism of action of hemorrhagic toxins on vascular endothelium is essential in this endeavor. Understanding such a mechanism might enable researchers to develop more suitable methods of prevention and treatment of the pathology connected with snake venom hemorrhagic toxins.

Previously, KURECKI and KRESS (1985) isolated an 85.7 kilodalton hemorrhagic toxin, proteinase H, from *C. adamanteus* venom. In the present study the effect of this toxin on the viability of rat aorta endothelial cell (RAEC), human dermal microvascular endothelial cell (HMVEC), and human umbilical

vein endothelial cell (HUVEC) monolayers was investigated using several viability assays. The methyl-thiazol-tetrazolium (MTT) assay has been used successfully to assay the cytotoxic effects of hemorrhagic toxins on murine capillary endothelial cells (LOMONTE *et al.*, 1994). This assay is based on the ability of living cells to reduce MTT to formazan via mitochondrial succinate dehydrogenase metabolism. This principle was first described in 1953 (BLACK AND SPEER) and was developed in the present form of the assay by MOSMANN in 1983. Its has the advantage of being rapid, reliable and objective, is very accurate, using a relatively small cell population, and delivers reproducible, linear results (FOULTIER *et al.*, 1992). Trypan blue dye uptake and lactate dehydrogenase release both assess the integrity of the plasma membrane as an index of cell viability. The trypan blue assay was used in one recent study to show that BaH1, a hemorrhagic toxin from the venom of *Bothrops asper*, was not cytotoxic to bovine endothelial cells (BORKOW *et al.*, 1995). In one other study of the effects of hemorrhagic toxins on endothelial cells, neutral red was used to assay the cytotoxicity of hemorrhagic toxins from *Crotalus ruber ruber* and *Crotalus atrox* on human vascular endothelial cells *in vitro* (OBRIG *et al.*, 1993*b*). The neutral red dye is taken up exclusively by viable cells and concentrated in lysosomes.

The collective results of these studies on the *in vitro* effects of hemorrhagic toxins on endothelial cells are inconclusive at best. Of the three studies published thus far there have been different results. However, each study employed different cell lines. Studies by LOMONTE *et al.* (1994) and BORKOW *et al.* (1995) showed that, while BaH1 did cause cell detachment, it had no direct cytotoxic effect on either immortalized murine capillary endothelial cells or bovine aortic endothelial cells. In the study by OBRIG *et al.* (1993*a*) HT-1 and HT-

2 from *C. ruber ruber* and HT-a and HT-d from *C. atrox* were all shown to be cytotoxic to HUVEC and human renal microvascular endothelial cells (HRMEC).

In the current study trypan blue dye exclusion, lactate dehydrogenase release, MTT metabolism and plating efficiency were used to assess the cytotoxic effects of proteinase H on HUVEC, HMVEC, RAEC, human intestinal smooth muscle cells (HISM), and sheep tendon fibroblasts (STF). Additionally, two non-hemorrhagic, non-proteolytic snake venom toxins, ACL myotoxin and myotoxin *a* were used as comparative controls, and trypsin was used as a non-hemorrhagic proteolytic control.

MATERIALS AND METHODS

Venoms and reagents

Crotalus adamanteus venom (lot CA/87A) was purchased from Biotoxins, Inc. (St. Cloud, FL, U. S. A.) in lyophilized form and kept at -20°C until use. Endothelial cell growth medium (EBM-MV) with BulletKit™ growth supplements was purchased from Clonetics Corp. (San Diego, CA, U. S. A.). Medium 199, lactate dehydrogenase assay kit 500-C, and endothelial cell growth supplement from bovine pituitary were purchased from Sigma Chemical Co. (St. Louis, MO, U. S. A.). Dulbecco's Modified Eagle's Medium (DMEM), 0.25 % trypsin, fetal bovine serum (FBS), amphotericin B (fungizone), penicillin-streptomycin solution, gelatin, heparin, L-glutamine, versene buffer, and 0.4 % trypan blue solution were purchased from Gibco BRL Life Technologies (Gaithersburg, MD, U. S. A.). The MTT assay kit was purchased from Chemicon (Temecula, CA, U. S. A.).

HMVEC cell culture

HMVEC obtained from Clonetics Corp. were seeded in Endothelial cell Basal Medium-Microvascular (EGM-MV) supplemented with 5 % FBS, 12 µg/ml bovine brain extract, 1 µg/ml cortisone, 10 ng/ml epidermal growth factor, 50 µg/ml gentamicin, 2.5 µg/ml amphotericin B (fungizone), 250 U/ml penicillin, 250 µg/ml streptomycin in sterile 24-well or 96-well culture dishes (Corning Glass, Corning, New York, USA) at a cell density of 5 X 10^3 cells/cm^2. Cells were fed with fresh medium every other day until reaching confluency within 8-10 days. Passages 4 and 5 were used for cytotoxicity studies.

HUVEC, RAEC, HISM, and STF cell culture

RAEC were the generous gift of Dr. Paula Grammas, Oklahoma University Health Sciences Center, Oklahoma City, OK, USA. STF were obtained as secondary cultures from Dr. Larry E. Stein, Oklahoma State University, Department of Physiological Sciences, College of Veterinary Medicine, Stillwater, OK, USA. HUVEC and HISM were obtained through American Type Culture Corporation (). RAEC, HISM, or STF were seeded either in 24-well or 96-well culture dishes (Corning Glass, Corning, New York, USA) at a density of 5.0 X 10^3 cells/ml^2 and cultured in 0.5 ml or 0.1 ml (24-well and 96-well dishes, respectively) DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.01 % (w/v) gentamicin, and 2.5 µg/ml fungizone (amphotericin B) at 37°C with 5 % CO_2. HUVECs were seeded and cultured in an identical conditions except the culture medium was Medium 199 with 10 % FBS, 2 mM L-glutamine, 10 µg/ml ECGS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 2.5 µg/ml fungizone. The cells were allowed to achieve confluency and experiments were conducted 8-12 days post-seeding. Culture

medium was changed every other day. For cytotoxicity assays HUVEC passages 12, 13 and 14 and RAEC passages 9, 10, and 11 were used.

Trypan blue and plating efficiency assays

Cells were cultured in 24-well dishes were washed twice with pre-warmed medium and then 250 µl of toxin mixed in culture medium was added to the appropriate wells. Culture medium with no toxin was used as a negative control, culture medium containing 0.1 % Triton X-100 was used as a positive cytotoxic control, and 0.25 % trypsin in PBS (pH 7.4) was used as a non-hemorrhagic, protease comparative control. After the appropriate incubation time the treatment medium was remove and the wells were washed with 250 µl of warmed HBSS to remove all detached cells. The HBSS wash was added to the treatment medium and centrifuged at 200 X *g* for 5 minutes. The resulting cell pellet was washed twice with HBSS and then re-centrifuged after each wash. The pelleted cells were resuspended in 500 µl of culture medium and half of these cells were counted using a Neubauer improved hemocytometer after the addition of 50 µl of 0.4 % Trypan blue. The percent cytotoxicity of the detached cells was expressed as the mean percent ± SEM of the total number of cells that were stained with trypan blue. The remaining cells were replated into sterile 24-well dishes.

The replated cells were allowed to attach for 6 hours and were washed with HBSS to remove any cells that did not reattach. The reattached cells were then trypsinized and both non-reattached and reattached cells counted as described above. Plating efficiency was calculated by dividing the number of reattached cells by the total number of cells and expressed as a mean percent of reattached cells ± SEM. Independent experiments were carried out in triplicate.

Lactate dehydrogenase (LDH) release assay

Cells were cultured and treated as described above for the trypan blue assay. Following treatment the medium was removed and replaced with plain culture medium to wash each well. The treatment medium was collected into 1 ml microfuge tubes and centrifuged at 200 X *g* for 5 minutes. The supernatant was carefully decanted and 100 µl assayed for LDH activity (LDH_r). The pelleted cells were lysed by the addition of 250 µl of culture medium containing 0.1 % triton X-100 and 100 µl of this was then assayed for LDH activity (LDH_d). The cells that remained in the culture vessels were washed as described and then lysed by the addition of 250 µl of culture medium containing triton X-100. Following lysis 100 µl of this mixture was assayed for LDH activity (LDH_a). The experiment was carried out in triplicate (n=3), and results were expressed as mean percent of total LDH released ± SEM:

$$\%LDH_{released} = \frac{LDH_r}{LDH_r + LDH_a + LDH_d}$$

MTT assay

Cells cultured in 96-well dishes were washed three times with 100 µl of pre-warmed HBSS prior to treatment to remove any detached cells, cellular debris, or metabolites that might interfere with the assay. Toxin was mixed with the appropriate culture medium, and 100 µl of this treatment medium was added to wells containing confluent cell monolayers and incubated at 37°C, 5 % CO_2. After the proper incubation time, 10 µl of a 5 mg/ml solution of MTT in PBS (pH 7.4) was added to each well and incubated for 4 hrs. at 37°C, 5 % CO_2 to allow cells to reduce the MTT to formazan. Following this incubation isopropyl alcohol with 0.04 N HCl (100 µl) was added to each well to stop the reaction and

to solubilize the formazan crystals. After mixing by repeated pipeting the absorbance of each well at 520 nm was read using a Thermomax ELISA plate reader (Molecular Devices, Menlo Park, CA, U. S. A) with 650 nm as a reference wavelength. The experiment was repeated in triplicate with three identical treatments per replicate (n=9). Cell culture medium with no toxin was used as a negative control, culture medium containing 0.1 % triton X-100 was used as a positive cytotoxic control. Comparative controls included 0.25 % trypsin, 0.5-5.0 µM ACL myotoxin, and 0.5-5.0 µM myotoxin *a*. Wells containing no cells were included as controls for each treatment to screen for possible artifacts in the MTT assay due to each treatment. The results were expressed as a mean percent of the negative control ± SEM.

Morphological changes and scanning electron microscopy

Changes in the morphology of cells were observed by phase-contrast microscopy and scanning electron microscopy. Cells used for SEM observation were cultured in 24-well dishes on sterile cover slips under the conditions described above. Following treatment cells were fixed in cacodylate buffered 2 % glutaraldehyde (pH 7.4) for 10 minutes. Following glutaraldehyde fixation cells were washed 3 times with 0.2 M cacodylate buffer (pH 7.4) for 5 minutes each wash. Cells were fixed with OsO_4 in cacodylate buffer for 10 minutes, washed with water 3 times, then dehydrated in a graded series of acetone (30 % - 100 %). Following the final dehydration step cells were critical point dried and then coated with gold-palladium for visualization by SEM. Cells were observed with a JEOL JSM-35U scanning electron microscope at 25 KV accelerating voltage.

Statistics

The data were analyzed by one-way ANOVA and Dunnet's *t*-test.

RESULTS

Cell detachment

Proteinase H caused HMVECs to detach from the substratum in a dose and time-dependent fashion (Fig. 1). This effect was slower than that seen with trypsinization but was nonetheless significant. Proteinase H at a concentration of 5 μM caused 76 ± 10 percent of cells to detach within 3 hrs. Nearly 100 percent of cells were detached within 6 hrs. after treatment with 5 μM proteinase H and 0.5 μM proteinase H caused nearly 100 percent detachment of cells within 24 hrs.

Trypan blue dye exclusion

Proteinase H caused treated HMVECs to take up trypan blue dye, indicating a compromise in plasma membrane integrity. Significant increases in the number of trypan blue-stained cells were observed following treatment of HMVECs with 5 μM proteinase H by 3 hrs, and by 6 hours with as little as 0.5 μM proteinase H (Fig. 2).

FIG. 1. HMVEC DETACHMENT INDUCED BY PROTEINASE H. Mean percentage of total cells detached from the bottom of culture wells after incubation with various concentrations of proteinase H (0.5 μM-5.0 μM). Treatments significantly different from control ($p<0.05$): 24 hrs at all concentrations; 6 hrs at 1, 3, and 5 μM; 3 hrs at 3 and 5 μM; 1 hr at 1, 3, and 5 μM.

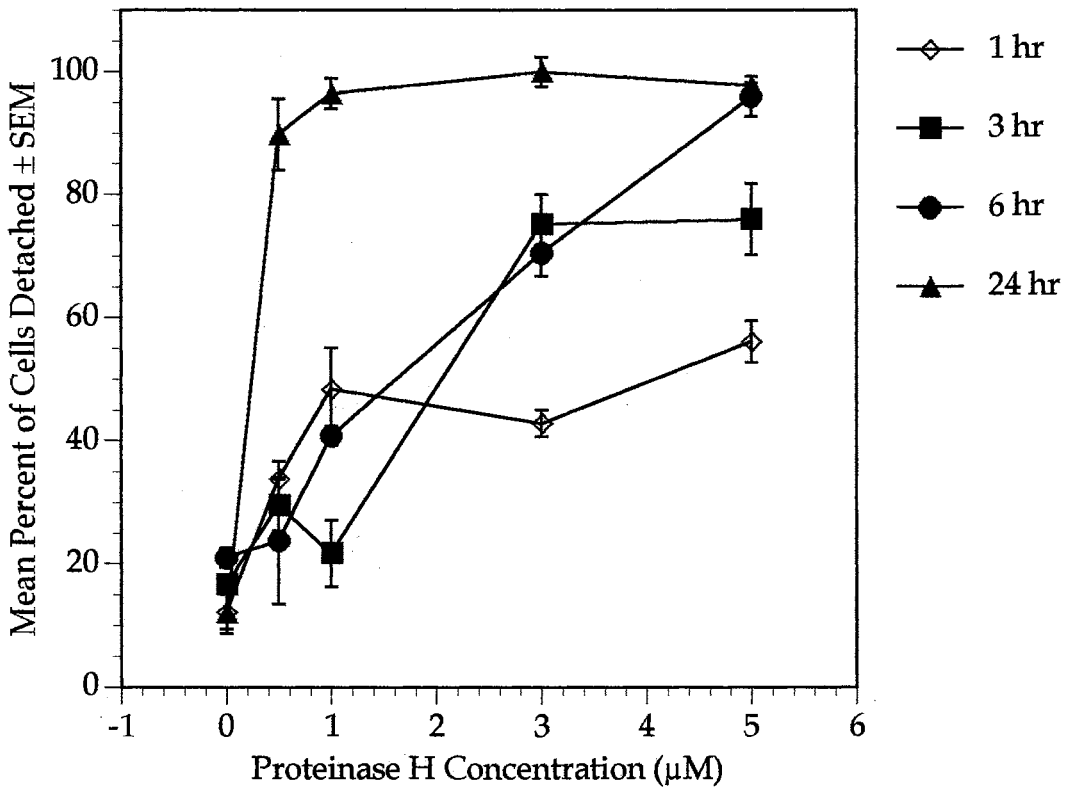
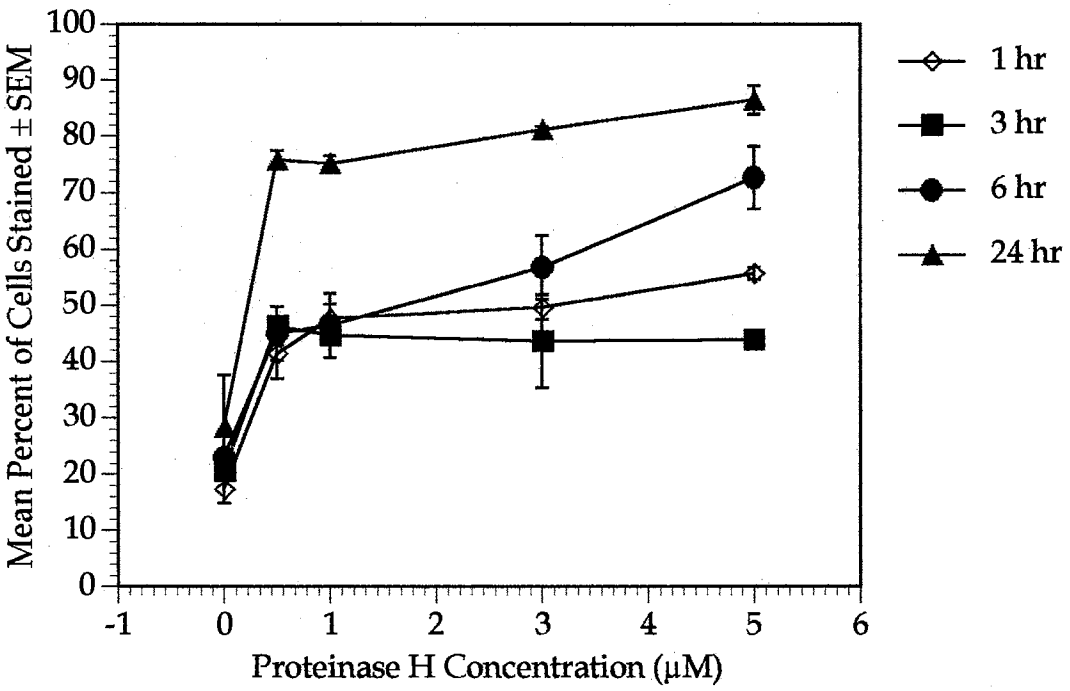

Mean Percent of Cells Detached ± SEM
100
80
60
40
20
0
-1
0
1
2
3
4
5
6
Proteinase H Concentration (μM)
1 hr
3 hr
6 hr
24 hr

FIG. 2. TRYPAN BLUE DYE UPTAKE IN HMVEC TREATED WITH PROTEINASE H. HMVEC were treated with proteinase H and attached and detached cells counted after the addition of 0.4 % trypan blue. Results presented here are expressed as the mean percent of the total cells per well stained with trypan blue for each treatment group. All treatments, 0.5 - 5.0 μM, were significantly different from control (0 μM) ($p<0.05$).


Mean Percent of Cells Stained ± SEM
100
90
80
70
60
50
40
30
20
10
0
-1
0
1
2
3
4
5
6
Proteinase H Concentration (μM)
1 hr
3 hr
6 hr
24 hr

Plating efficiency assay

Treatment of HMVEC with proteinase H caused cells to detach from the substratum and compromised the ability of these detached cells to reattach following washing with culture medium as evidenced by a significant decrease in their plating efficiency (Fig. 3). It should be noted that the actual number of cells that were detached was much greater in the proteinase H-treated groups compared to the non-treated control. Of those detached cells in the control groups nearly half of them reattached within 6 hrs. In the groups treated with proteinase H the percent of reattachment of cells ranged from a low of about 5 % (5 μM, 24 hrs.) to a high of about 46 % (0.5 μM, 24 hrs).

Lactate dehydrogenase release assay

Proteinase H at concentrations up to 5 μM failed to cause LDH release from HISM, STF, RAEC or HUVEC. Proteinase H caused significant release of LDH from HMVEC at concentrations as low 0.5 μM after 24-hours (Fig. 4). No significant increase in LDH release was observed at earlier time periods.

MTT assay

Treatment of HMVEC with proteinase H caused a decrease in the MTT-reducing ability within 6 hrs. at a concentration of 5 μM and within 24 hrs at 0.5 μM (Fig. 5). Proteinase H did not affect the MTT-metabolizing ability of the other cell types tested.

Morphological changes

Following treatment with proteinase H holes began to appear in the HMVEC monolayer within 1 hr (Fig. 6). At later time periods, within 3 hrs.,

FIG. 3. EFFECT OF PROTEINASE H ON PLATING EFFICIENCY OF HMVEC. Detached cells following proteinase H treatment were washed with fresh culture medium and then replated. After 6 hrs. cells which remained unattached were collected and counted. Cells that reattached were trypsinized and then counted. Plating efficiency is expressed as the mean percent of the total detached cells (unattached cells/total detached cells) that were able to reattach after 6 hrs.

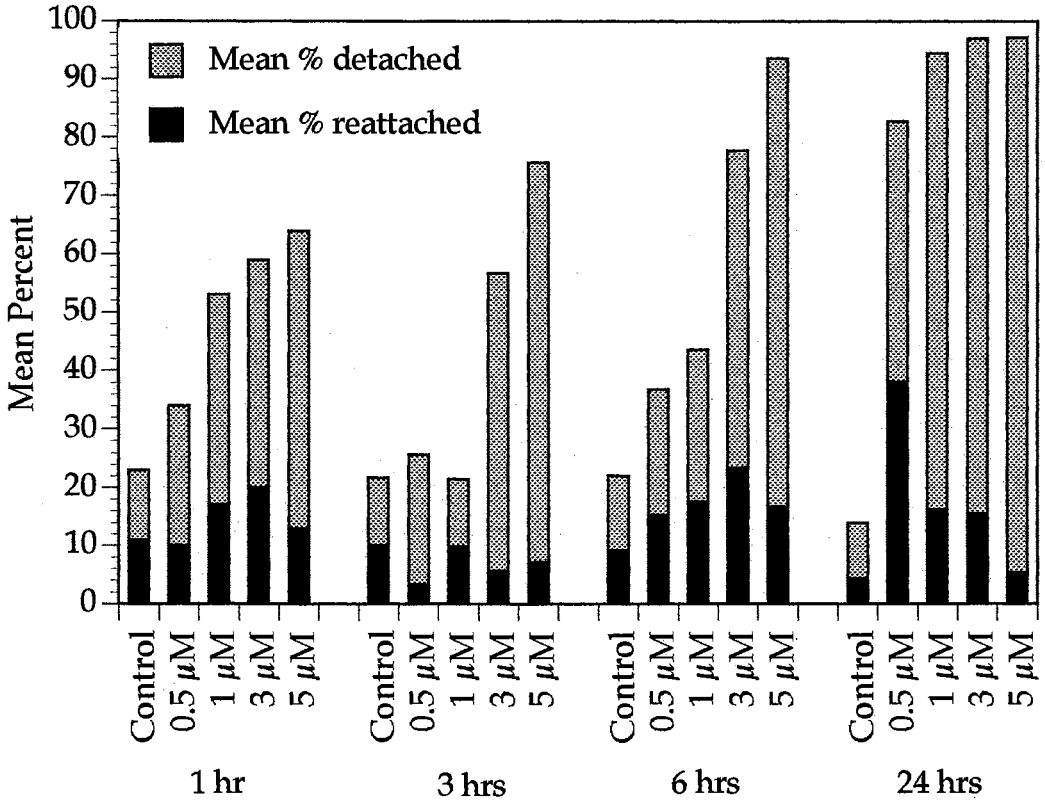

Mean % detached
Mean % reattached
Mean Percent
100
90
80
70
60
50
40
30
20
10
0
Control
0.5 µM
1 µM
3 µM
5 µM
1 hr
3 hrs
6 hrs
24 hrs

FIG. 4. LDH RELEASE ASSAY OF HMVEC TREATED WITH PROTEINASE H. Compared to non-treated controls, proteinase H caused significant ($p<0.05$) release of LDH from HMVEC *in vitro* within 24 hrs at concentrations greater than or equal to 0.5 μM.

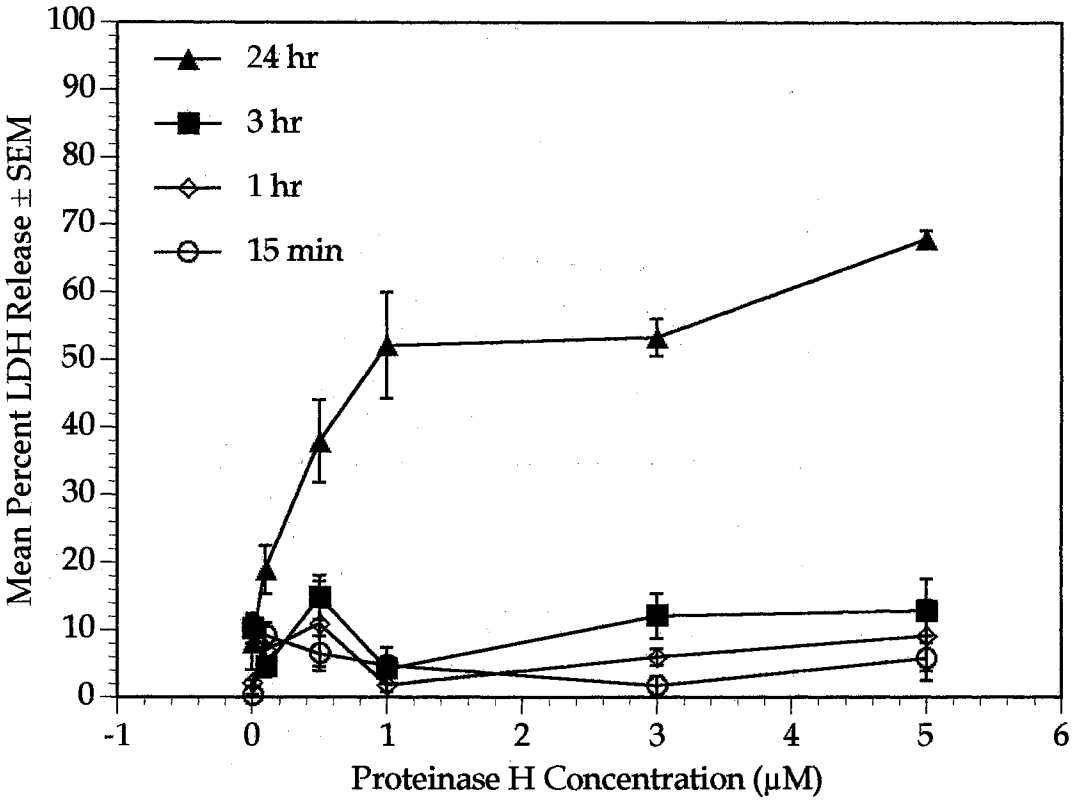

24 hr
3 hr
1 hr
15 min
Mean Percent LDH Release ± SEM
Proteinase H Concentration (µM)
100
90
80
70
60
50
40
30
20
10
0
-1
0
1
2
3
4
5
6

FIG. 5. EFFECT OF PROTEINASE H ON MTT-REDUCING ABILITY OF HMVEC. Proteinase H caused a significant ($p<0.05$) decrease in the MTT-reducing ability of HMVEC within 6 hours at 5 μM and within 24 hrs at 0.5 μM or greater concentrations when compared to controls.

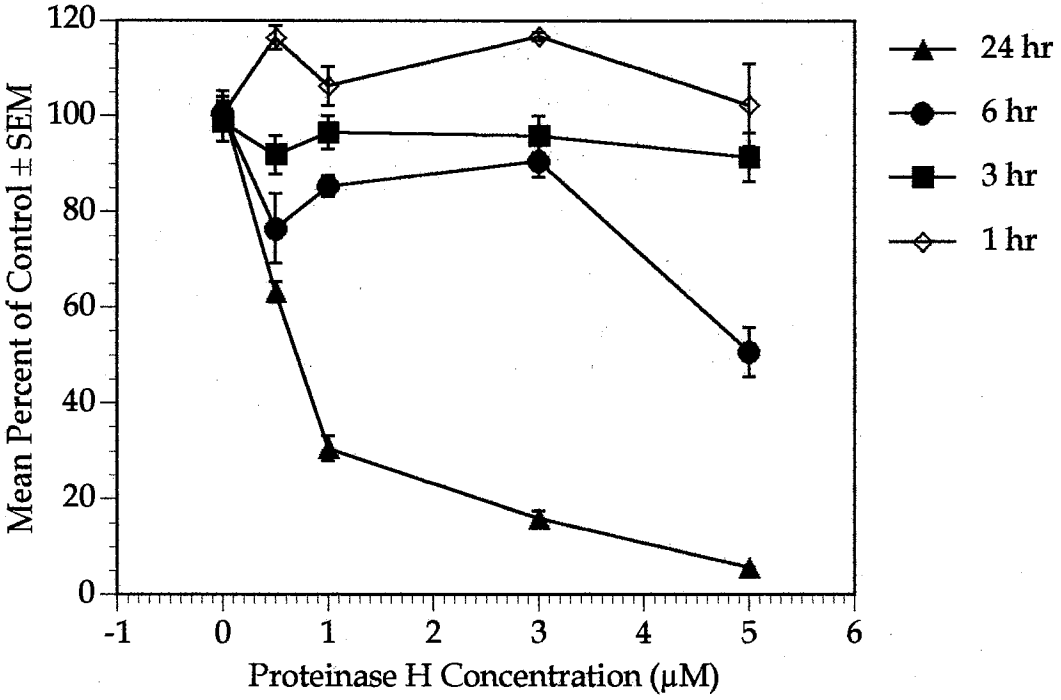

120
100
80
60
40
20
0
-1
0
1
2
3
4
5
6
Mean Percent of Control ± SEM
Proteinase H Concentration (μM)
24 hr
6 hr
3 hr
1 hr

FIG. 6. MORPHOLOGICAL CHANGES IN HMVEC INDUCED BY PROTEINASE H. Cells were treated with 1 μM proteinase H for various periods of time. (A) Control cells after 24 hrs. (Bar = 50 μm.) Proteinase H-treated cells after (B) 1 hr (Bar = 50 μm.); (C) 3 hrs (Bar = 50 μm.); D 24 hrs. (Bar = 50 μm.)

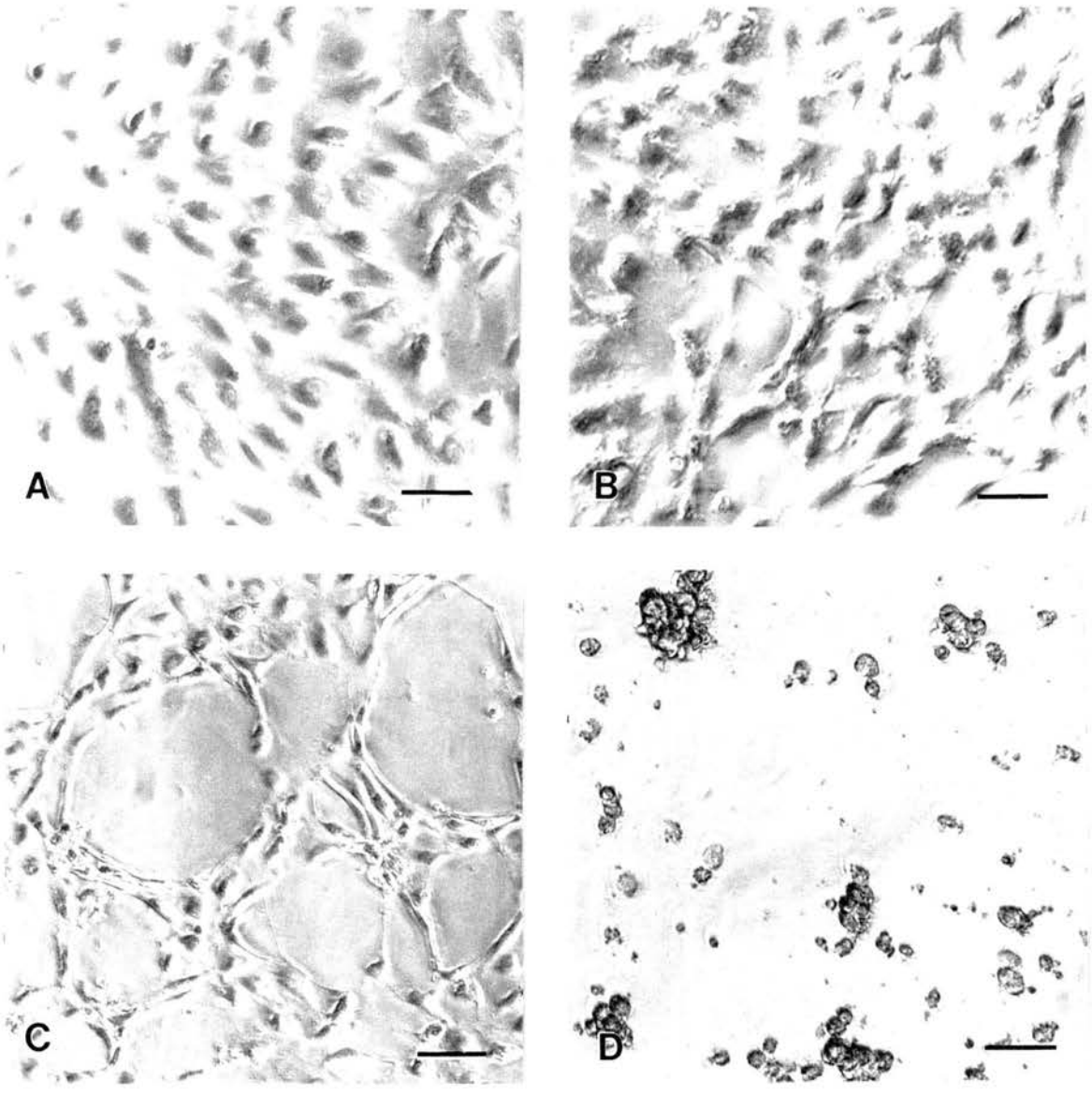

A
B
C
D

FIG. 7. MORPHOLOGICAL CHANGES IN HUVEC INDUCED BY PROTEINASE H. Cells were treated with 1 μM proteinase H for various periods of time. (A) Control cells after 24 hrs. (Bar = 50 μm.) Proteinase H-treated cells after (B) 1 hr (Bar = 50 μm.); (C) 3 hrs (Bar = 50 μm.); D 24 hrs. (Bar = 20 μm.)

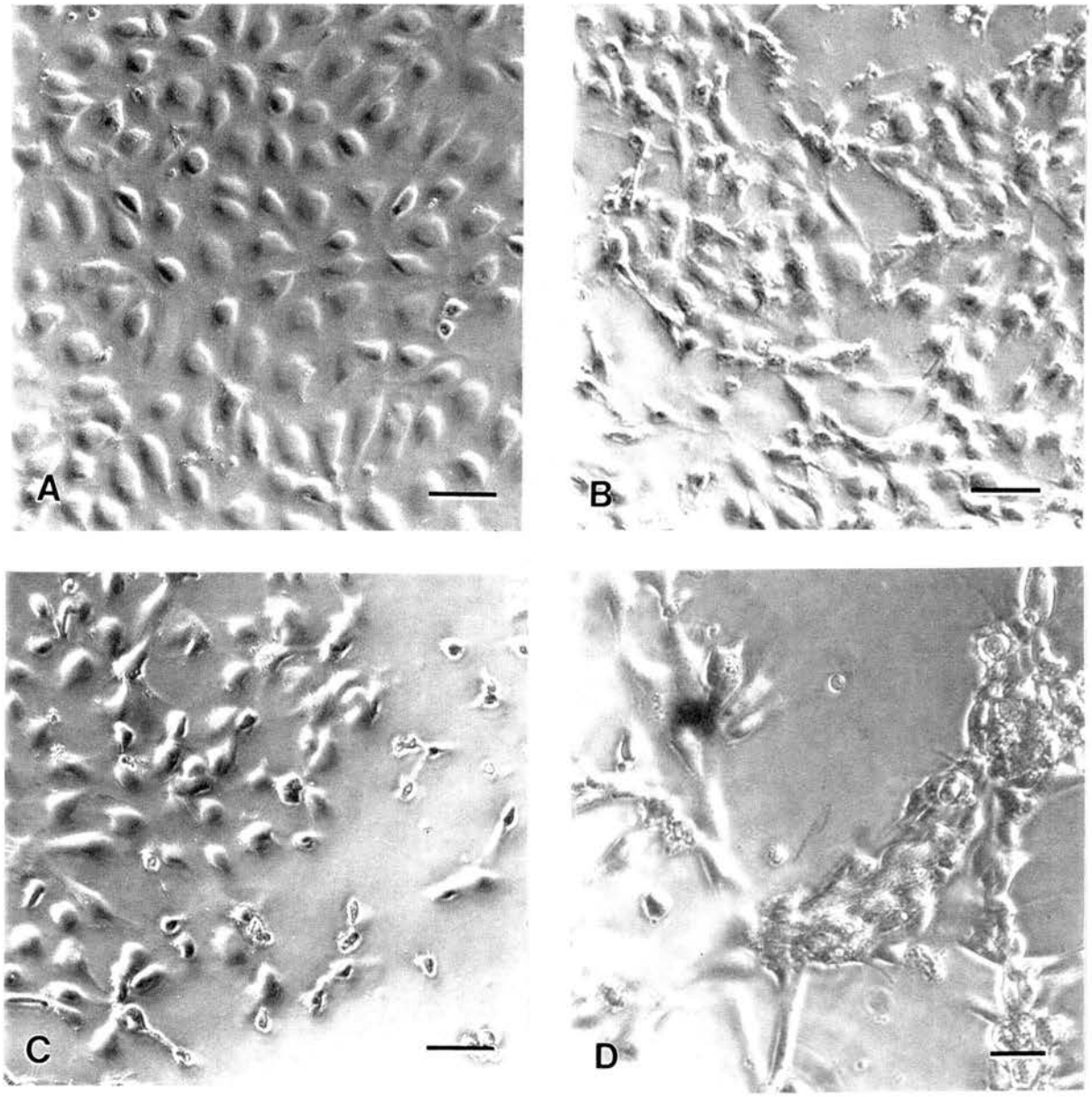

A
B
C
D

FIG. 8. SCANNING ELECTRON MICROGRAPH OF PROTEINASE H-TREATED HMVEC. Cells treated with 3 μM proteinase H were analyzed for morphological changes after 12 hrs. (A) Control cells. (Bar = 100 μm.); (B) proteinase H-treated cells. (Bar = 100 μm.)

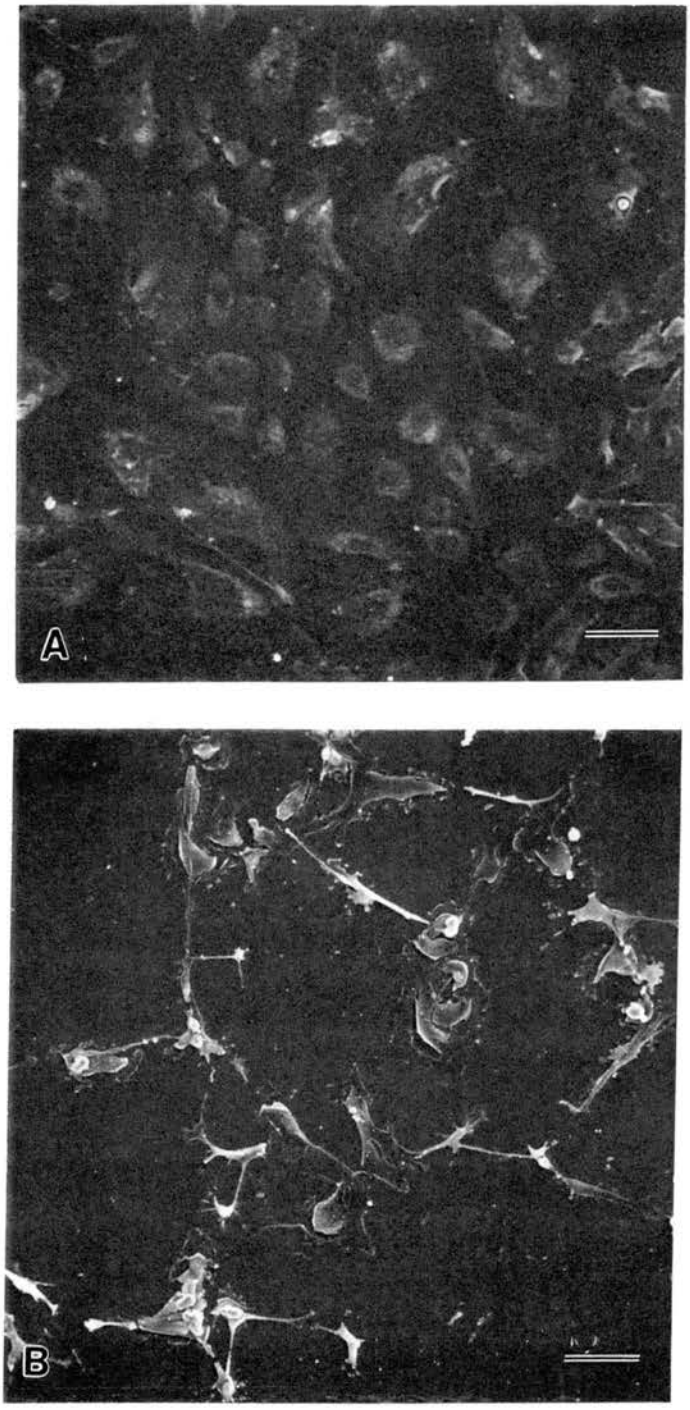
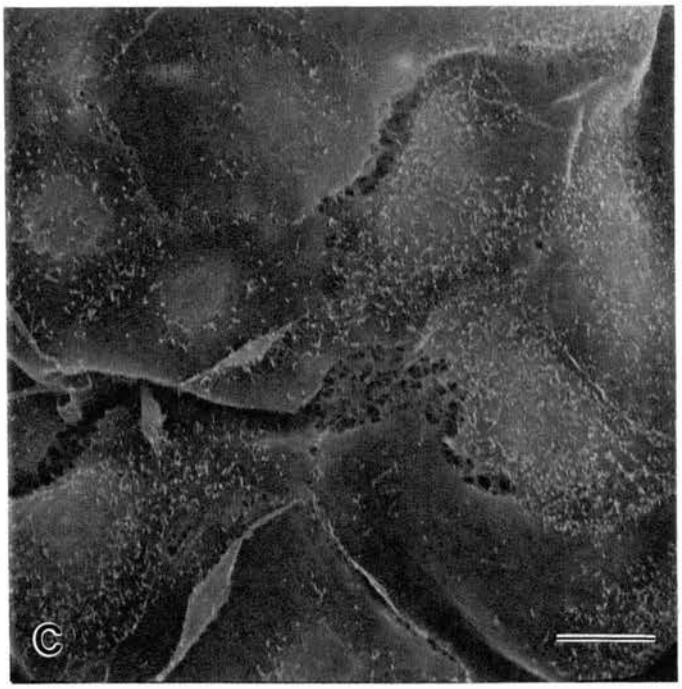
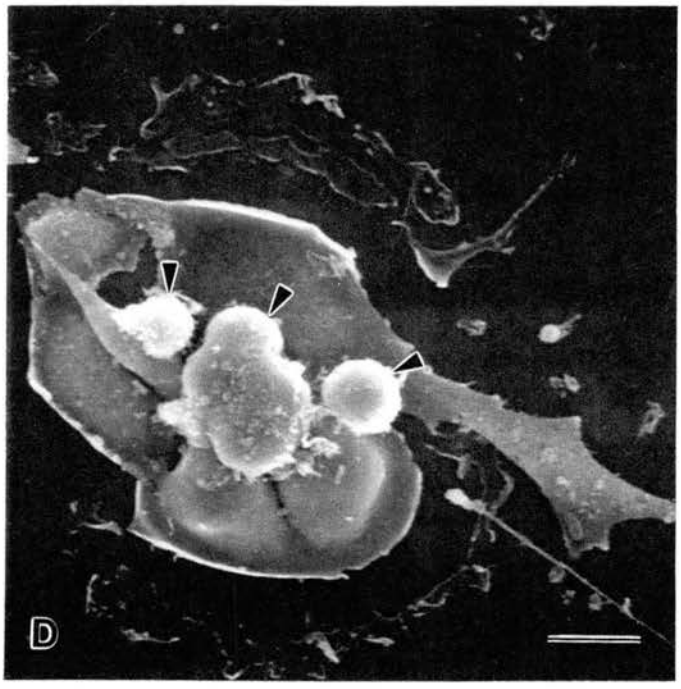

A
B

FIG. 8 (cont'd). C, control cells. (Bar = 10 μm.) D, proteinase H-treated cells showing blebbing of the plasma membrane (arrowheads). (Bar = 20 μm.)


C


D

cells were completely rounded and detached from the substratum. Similar morphological changes were observed in proteinase H-treated HUVEC (Fig. 7). SEM revealed evidence of cellular damage in the form of plasma membrane blebbing (Fig. 8). Whereas both HUVEC and HMVEC exhibit similar changes in the monolayer specific cellular alterations were observed only in the HMVEC groups. This suggests that in both cell types a "non-specific" action on the extracellular substratum and a much more "cell-specific" effect only on the HMVEC.

DISCUSSION

Endothelial cell damage has been elicited by a variety of substances including hemoglobin (FOLEY *et al.*, 1994), free radicals such as hydroxyl anions (VARANI *et al.*, 1985), H_2O_2 (WHORTON *et al.*, 1985), and nitric oxide (VOLK *et al.*, 1995), neutrophil elastase (WESTLIN and GIMBRONE, 1993), cyclophosphamide (KACHEL and MARTIN, 1994), β-VLDL (TANIMURA *et al.*, 1990), lipopolysaccharide (MCGRAT and STEWART, 1969; HARLAN *et al.*, 1983), bacterial toxins such as Shiga toxin (KAYE *et al.*, 1993), toxic shock syndrome toxin 1 (LEE *et al.*, 1991), and a host of other agents including some snake venom hemorrhagic toxins (OBRIG *et al.*, 1993*b*). General characteristics of damage to endothelial cell monolayers *in vitro* include cell detachment, retraction and plasma membrane blebbing, release of endothelial cell markers such as thrombomodulin (SAWADA *et al.*, 1992), angiotensin converting enzyme (MATUCCI-CERINIC *et al.*, 1992) and von Willebrand factor (BLANN, 1993), and changes in the release and expression of various cytokines and cell adhesion molecules (GRACE, 1994).

The results of the current study indicate that proteinase H is directly cytotoxic to HMVEC *in vitro*. Proteinase H did not exert a cytotoxic effect on the

other cell types tested. As proteinase H is a proteolytic enzyme its actions were compared to that of a non-hemorrhagic, general protease, trypsin. When cells were treated with 0.25 % trypsin for 1 hr to 24 hrs cell cytotoxicity determined by either LDH release or trypan blue dye uptake was comparable to or greater than that resulting from proteinase H treatment. Trypsin has long been known to damage cells due to its proteolytic activity (FRESHNEY, 1987). However, in the current study trypsin did not significantly decrease the ability of HMVEC to metabolize MTT nor did it significantly decrease the plating efficiency of HMVEC. In addition, proteinase H was selectively cytotoxic only to HMVEC while trypsin acted on and damaged indiscriminately all the cell types employed in the study. Moreover, similar changes were observed in both HMVEC and HUVEC monolayers (i. e. appearance of holes in the monolayer) while only in HMVEC were morphological changes observed in individual cells. Furthermore, trypsinization resulted in the release from the substratum and damage of all the cell types thus treated while proteinase H had absolutely no discernible effect on RAEC, HISM, or STF. If the action of proteinase H were simply proteolytic degradation one would expect some degree of alteration or damage to be seen in all the cell types tested and this was not the case.

Taken together, these findings suggest that the action of proteinase H against HMVEC is more than just simple proteolytic damage. These findings demonstrate that proteinase H has a specific cytotoxic activity against HMVEC and not against arterial or venous endothelial cells *in vitro*. Cell-specific and species-specific variations in the proteins comprising the extracellular substratum of or differences in the cell-surface proteins expressed by each cell type may explain the selectivity of proteinase H for HMVEC. Also, proteinase H may be less active against HUVEC, and greater concentrations of proteinase H might elicit the same changes in HUVEC as seen in HMVEC. These findings are

consistent with data from studies showing phenotypic heterogeneity in endothelial cell populations from different vessels, within the same vessels and in different tissue sites (AUERBACH, 1992; TOMLINSON *et al.*, 1991). Such heterogeneity has even been reported in the expression of cell-surface receptors for bacterial toxins (OBRIG *et al.*, 1993*a*)

The early appearance of spaces and detachment of HMVEC is consistent with findings by other authors using bovine (BORKOW *et al.*, 1995) and murine endothelial cells (LOMONTE *et al.*, 1994) and supports the idea that disruption of the capillary basement membrane is one primary effect of snake venom hemorrhagic toxins. The death of capillary endothelial cells as seen in this study may be secondary to the disruption of the basement membrane, but evidently involves more than simple proteolysis in that trypsin does not result in the same effect on HMVEC.

In summary, treatment of human dermal capillary endothelial cells (HMVEC) with proteinase H causes the morphological changes (i. e. detachment, retraction, blebbing) associated with cell damage as well as membrane disruption and metabolic disturbances as evidenced by LDH release and trypan blue uptake and decreased MTT reduction respectively. HMVEC plating efficiency was also significantly decreased by treatment with proteinase H. These changes were not observed in the other cell types tested. These data lead us to conclude that proteinase H is directly cytotoxic to HMVEC cells and not to HUVEC, RAEC, HISM, or STF *in vitro*.

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CHAPTER VII

DISCUSSION

Summary

The purpose of the preceding work was to demonstrate the biochemical characteristics and biological actions of the hemorrhagic toxin, proteinase H. In particular, the local and systemic effects of proteinase H in mice and the effects of proteinase H on cultured endothelial cells were studied.

Proteinase H was found to be a large (MW = 85.7 kD), metalloproteolytic glycoprotein comprising an estimated 0.7 % of the crude venom (Chapter III). It exhibited proteolytic activity against Azocoll™, *N,N*-dimethylated casein, and fibrinogen, accounting for approximately 4.8% of the total proteolytic activity of the venom. Proteinase H also exhibited a hemorrhagic activity with a minimum hemorrhagic dose of 0.02 μg/g (Chapter II). Both the proteolytic and hemorrhagic activities of proteinase H were inhibited by EDTA and by deglycosylation by treatment with PNGaseF (Chapter III).

Proteinase H was found to have a relatively carbohydrate content (Chapter III). Deglycosylation of proteinase H with the *N*-glycosidase PNGaseF resulted in the total removal of all glycosyl moieties detectable by PAS staining or lectin blotting. In lectin blotting assays it was also found that proteinase H is labeled with *Datura stramonium* agglutinin (DSA) which is specific for glycosyl residues containing the disaccharide galactose-β(1-4)-*N*-acetylglucosamine in complex and hybrid *N*-glycans. Removal of the glycosyl moieties of proteinase

H resulted in a decrease in its molecular weight to 50.6 kD as estimated by SDS-PAGE. This estimation was corroborated by amino acid analysis which found that proteinase H contained 355 residues and had a calculated molecular weight of 49.4 kD. Inhibition of the enzymatic and hemorrhagic activity of proteinase H by deglycosylation indicated that the glycosyl moieties play and important role in the activity of proteinase H (Chapter III).

Proteinase H induced the formation of fibrin clots from bovine fibrinogen by release of fibrinopeptide B and subsequent degradation of the Bβ subunit of fibrinogen (Chapter II). The clotting activity of proteinase H was estimated to be equivalent to 5.0 NIH U/mg protein. The clots thus formed differed visibly from those formed by treatment of fibrinogen with thrombin in that they were more opaque and collapsed to an insoluble precipitate upon vigorous vortexing. The characteristics of proteinase H-induced clots were similar to those reported by DOWD *et al.* (1995) for clots formed by treatment of fibrinogen with cathepsin-L2 from *Fasciola hepatica,* the only other non-serine proteinase reported to cause fibrinogen-clotting. Interestingly, the fibrinogen-clotting activity of proteinase H is not inhibited by EDTA or by deglycosylation (Chapter II). These are paradoxical findings in that both of these treatments inhibit the proteolytic and hemorrhagic activities of proteinase H. The only explanation we were able to derive from these data is that proteinase H is able to retain some highly specific, residual proteolytic activity with which it is able to cause the release of fibrinopeptide B, leading to fibrinogen clotting.

Studies on the pathological effects of proteinase H were also conducted (Chapter IV). It was thus determined that the administration of proteinase H via intramuscular injection resulted in local hemorrhage *per rhexis.* Proteinase H affected endothelial cells directly, causing cell swelling, mitochondrial swelling, ER dilatation, cell membrane blebbing and rupture of the plasma membrane.

Hemorrhage was rapid (within 10 min.) and extensive, extending over the entire thigh, but platelet plug formation and fibrin deposition were not observed. Associated with hemorrhage was myocyte damage (i. e., swelling, SR dilatation) and myonecrosis within 1 hr at a dose of 5 μg/g. Inflammation primarily in the form of neutrophil infiltration was also closely associated with the onset of myonecrosis. At 24 hrs after injection both myonecrosis and inflammation were severe.

To further the understanding of the role of proteinase H in the pathology of rattlesnake envenomation, *in vivo* studies were conducted to determine what systemic effects proteinase H induced in mice (Chapter V). Following intravenous injection of proteinase H (5 μg/g) severe hemorrhage was observed in many internal organs including the heart, lungs, stomach and small intestine. At this dose mice died within 3 hrs. Glomerulonephropathy and hepatic degeneration were also observed. Renal glomeruli were swollen and congested. Damage to the glomerular endothelium in the form of cell and mitochondrial swelling and ER dilatation was observed by transmission electron microscopy. Endothelial cells remained intact and the basal laminae were not visibly affected. In the liver an enhanced lobular pattern was observed grossly at 3 hrs following earlier signs of edema. Histologically, glycogen depletion of hepatocytes occurred within 1 hr and by 3 hrs many hepatocytes were vacuolated and contained pyknotic nuclei. Sinusoidal endothelium and hepatic vascular structures were not noticeably affected. It was concluded that these changes in the kidney and the liver were most likely secondary events due to ischemia and/or hypoxia brought about as a result of severe pulmonary and cardiac hemorrhage. However, the direct action of proteinase H on these tissues cannot be ruled out.

The effect of proteinase H on cultured cells was determined using fibroblast, smooth muscle and endothelial cell lines (Chapter VI). It was thus determined that proteinase H has no effect on sheep tendon fibroblasts (STF), human intestinal smooth muscle cells (HISM), or rat aorta endothelial cells (RAEC). Proteinase H does induce morphological changes in human umbilical vein endothelial cell (HUVEC) monolayers, causing holes to form in these monolayers, but is not cytotoxic to HUVEC. Similar morphological changes in HUVEC and murine capillary endothelial cells have been reported for other hemorrhagic toxins and are thought to be the result of proteolytic degradation of endothelial basal lamina proteins. Proteinase H caused a similar morphological change in human dermal microvascular endothelial cells (HMVEC) but was also found to be cytotoxic to these cells. Proteinase H induced cell retraction, rounding and detachment and membrane blebbing of HMVECs and caused cell membrane damage that was assessed by trypan blue dye uptake and LDH release assays. Proteinase H treatment also led to the loss of mitochondrial reduction of MTT, a further indication of the cytotoxic effect it rendered upon HMVECs. These findings correlated well with *in vivo* observations that proteinase H selectively damaged capillary endothelial cells to cause hemorrhage *per rhexis*.

Conclusions

The first hypothesis of this dissertation was that proteinase H activity (proteolytic and hemorrhagic) is dependent upon glycosylation. The observation that removal of glycosyl residues inhibits/abolishes both of these activities allows this hypothesis to be accepted. The exact nature of the role of glycosyl moieties cannot be determined from these observations, but inferences can be drawn from other studies. I propose that the primary role of the

glycosylation of proteinase H is to provide molecular stability for the maintenance of an enzymatically active conformation. This is supported by the fact that both EDTA-treatment and deglycosylation have the same inhibitory effect on proteinase H. As mentioned previously (Chapter II) proteinase H is a zinc-dependent metalloproteinase belonging to the metzincin superfamily of metalloenzymes. It thus shares certain structure/function characteristics with fibrolase, a fibrinolytic enzyme belonging to the same family of enzymes. PRETZER *et al.* (1992) demonstrated, by analysis of circular dichroism spectra, that removal of zinc from fibrolase resulted in a conformational change in the molecule. Specifically, a decrease in the content of alpha helices in the molecule was observed. This decrease was interpreted as the uncoiling of the alpha helix containing the enzymatic active site of fibrolase. Thus, removal of a divalent metal cation, zinc, resulted in molecular instability leading to the loss of enzymatic activity. VAN HUYSTEE and WAN (1994) reported similar changes in a different enzyme, peanut peroxidase, after deglycosylation. In that study it was reported that removal of *N*-linked sugars by PNGaseF resulted in the loss of a calcium ion from the enzymatic active site of peanut peroxidase. This was associated with loss of alpha helical content of the molecule which was interpreted as the uncoiling of the enzymatic active site. In both of these cases the loss of a metal cation from the active site is associated with loss of alpha helical content and concurrent loss of enzymatic activity. Deglycosylation is related to the loss of a divalent metal cation in certain metalloenzymes. It can thus be inferred that, in particular enzymes, molecular stability is associated with glycosylation. Proteinase H is such an enzyme.

Microvascular hemorrhage can occur via two routes. In hemorrhage *per rhexis,* the endothelial cells forming capillaries are directly lysed and hemorrhage occurs by virtue of this microvascular disruption. In hemorrhage

per diapedesis the intracellular junctions of endothelial cells are broken allowing extravasation of blood components to occur intracellularly. In the current study it has been clearly demonstrated that proteinase H induces hemorrhage *per rhexis*. This confirms the second hypothesis of the dissertation. This finding, taken together with the observation of systemic hemorrhage, is important in considering the actual mechanism by which proteinase H induces hemorrhage. The dramatic pulmonary, cardiac, and gastrointestinal hemorrhage after intravenous injection strongly suggests that proteinase H directly lyses the capillary endothelium in these organs. This is particularly apparent in considering the lungs. The pulmonary microvasculature is a characteristically tight barrier. As such, proteinase H, a relatively large protein, must cross the endothelial cell barrier to act on the endothelial basal lamina. It is doubtful that it is able to do so, yet it still induces hemorrhage *per rhexis* in the lungs. Thus, proteinase H directly lyses the vascular endothelium.

Proteinase H appears to be partially selective for the pulmonary endothelium. This is particularly obvious when considering that intravenous administration of proteinase H induced very severe hemorrhage in the lungs without inducing any hemorrhage in skeletal muscle tissue. It is known that proteinase H is able to induce hemorrhage in skeletal muscle after intramuscular injection. After intravenous injection the pulmonary and skeletal muscle vasculature are both exposed to proteinase H. This leads one to conclude that proteinase H "prefers" the pulmonary, cardiac, and GI microvasculature. This is most likely due to phenotypic heterogeneity of different endothelial cell populations determined by their location within the body. This kind of heterogeneity is well documented (AUERBACH, 1992; HEWETT and MURRAY, 1993; PETZELBAUER *et al.*, 1993). OBRIG *et al.* (1992) even demonstrated this kind of phenotypic heterogeneity regarding the expression of cell surface receptors

specific for a bacterial toxin. The evidence described above would suggest that a cell surface receptor may exist for proteinase H on certain microvascular endothelial cells and that the expression of this receptor varies between endothelial cell populations. If there is such a receptor, the microvascular endothelium of skeletal muscle clearly expresses it, but proteinase H seems to prefer the microvascular endothelium of other organs when administered intravenously. This is an additional possible role of the glycosyl moieties of proteinase H: the function of ligand recognition and/or binding.

The third hypothesis of the dissertation, that proteinase H is directly cytotoxic to microvascular endothelial cells, was also supported by the findings of the study. Proteinase H was directly cytotoxic to cultured HMVEC (Chapter VI). More importantly, proteinase H was <u>not</u> cytotoxic to sheep tendon fibroblasts, human intestinal smooth muscle cells, rat aorta endothelial cells, nor human umbilical vein endothelial cells. The predominant theory regarding the action of snake venom hemorrhagic toxins is that they digest the endothelial basal lamina to induce hemorrhage *per rhexis*. As mentioned above, it seems clear that proteinase H induces hemorrhage *per rhexis* by direct lysis of endothelial cells. In the cell culture studies cells were plated on plastic and were grown without the assistance of pre-coating vessels with an artificial "basal lamina". Proteinase H was still cytotoxic to HMVEC, suggesting that it is not necessary to digest the basal lamina to induce cell lysis. The initial response of HMVEC (and HUVEC) to proteinase H was retraction. This seems to suggest that the cells respond directly to the presence of proteinase H. Additionally, proteinase H was cytotoxic after application to the "lumenal" surface of cultured HMVEC, further evidence that the basal lamina need not be involved.

According to the Handbook of Toxinology there are seven different classes of cytotoxins (SHIER and MEBS, 1990). These include cytolytic toxins,

toxins that inhibit protein synthesis, enterotoxins, toxins altering genetic expression, toxins acting on the cytoskeleton, toxins acting on ion channels and synapses, and bacterial endotoxins. Due to mechanistic considerations and differences in molecular structures I do not think that proteinase H can be reasonably considered as belonging to any of these classes of cytotoxins except possibly for cytolytic toxins and enterotoxins.

There are four subclasses of cytolytic toxins: pore-forming toxins, cytolytic enzymes, detergent-like toxins, and toxins acting via unknown mechanisms. Pore-forming toxins come in a variety of sizes, isoelectric points and molecular structures. It is conceivable that proteinase H acts via this mechanism, although there is no data to support this. Cytolytic enzymes directly destroy the plasma membrane or make the plasma membrane more susceptible to lysis by enzymatic digestion of membrane phospholipids. Thus, these toxins are phospholipases. There is no evidence to suggest that proteinase H is a phospholipase, so I believe that proteinase H is not a cytolytic enzyme. Detergent-like toxins are generally non-protein, surfactant molecules. It is unlikely that proteinase H operates via this mechanism. At present, if proteinase H were included as a cytolytic toxin it would most likely be classified as one with an unknown mechanism.

Enterotoxins act on the digestive tract and inhibit water absorption primarily by disrupting ion transport in gut epithelial cells via a G-peptide dependent receptor-mediated mechanism. Proteinase H can obviously be excluded from this class of toxins in that it does not induce the same physiological changes as enterotoxins. However, proteinase H may act on endothelial cells via a similar mechanism of action, i. e., receptor-mediated cytotoxicity. In fact, one enterotoxin, Shiga toxin, is a relatively large protein (70 kD) that has been shown to bind to cell surface glycolipid receptors on

endothelial cells *in vitro* (OBRIG *et al.*, 1993). The mechanism of action of Shiga toxin is not clear but the end result is a reduction in cell viability and protein synthesis. The cytotoxic effect of proteinase H against HMVEC *in vitro* follows a similar time course as that shown for Shiga toxin against HUVEC and human renal microvascular endothelial cells (HRMEC) *in vitro*. As mentioned in Chapter VI, this effect is much slower than hemorrhage actually occurs *in vivo*. Shiga toxin cytotoxicity was enhanced by the addition of tumor necrosis factor (TNF) indicating that there are other factors involved in its *in vivo* activity. This may also be true for proteinase H.

Another interesting observation regards the role of proteolysis in the activity of proteinase H. If the action of proteinase H is due to its proteolytic activity one might expect it to have the same effect on all cells, much like trypsin. This was not the case, and there are three explanations that come readily to mind. First, the proteolytic activity of proteinase H may not play a role in its cytotoxic activity. This would not be entirely surprising as proteinase H is not highly proteolytic on any of the substrates it has been tested against when compared to trypsin, pronase, or other proteases. If the proteolytic activity of proteinase H is not involved in cytotoxicity then what is its role? The answer to this question is elusive. A second, and perhaps more plausible, explanation is that the proteolytic activity of proteinase H is highly specific for proteins produced by certain microvascular endothelial cells. This would explain the preferential cytolytic effect of proteinase H *in vivo* as well as the selective cytotoxic effect of proteinase H *in vitro*. The third explanation is that the cytotoxic activity of proteinase H is due to the presence of cell surface receptors on certain microvascular endothelial cells.

Either of the latter two hypotheses of the selective cytotoxic effect of proteinase H might also explain the *in vivo* observations of

glomerulonephropathy and proximal convoluted tubule swelling. Proteinase H was found to induce damage to glomerular endothelial cells and congestion of glomerular capillaries. Initially one would expect that glomerular damage would be due to hypoxic ischemia as a result of decreased vascular perfusion and decreased oxygenation of erythrocytes because of pulmonary and cardiac hemorrhage. Instead, glomerular capillaries were hyperemic. Thus the glomeruli were well-perfused. This paradoxical observation suggests that the glomeruli were directly damaged. A similar conclusion may be drawn from the swelling of the proximal convoluted tubular epithelium. Hepatocyte degeneration is most likely due to ischemic conditions in the liver, but a direct toxic effect of proteinase H cannot be ruled out. Hypothetically, proteinase H may be toxic to glomerular endothelial cells, proximal convoluted tubule epithelial cells and hepatocytes. If this is the case, it would appear that a common receptor or target molecule exists on all the cell types affected. One weakness in the work conducted to asses the toxic affects of proteinase H *in vivo* is that, except for skeletal muscle, only tissues that were visibly affected were selected for microscopic observation. Because of this it cannot be concluded that proteinase H was not cytotoxic to cells in other tissues. Future studies might address this.

Proteinase H also exhibited other characteristics that are most interesting such as the observation that proteinase H induces fibrinogen-clotting. It should be emphasized that this activity was not demonstrated *in vivo*. If it does actually occur *in vivo*, the role of this activity, if any, in the function of proteinase H as a hemorrhagic toxin is uncertain. Snake venom procoagulants have previously been implicated in the induction of hemorrhage by virtue of their fibrinogen-depleting action. Such agents induce the slow formation of fibrin to form microthrombi which do nothing to promote hemostasis but instead compromise

the clotting ability of the blood. This is a likely explanation of the fibrinogen-clotting activity of proteinase H.

A final observation about the *in vivo* activity of proteinase H is the conspicuous absence of platelet plugs in intact vessels following treatment with proteinase H. Unfortunately, it is impossible to conclude that platelet plugs definitely do not form because they may simply not be present in the tissue samples that were analyzed. They may have been present in other samples that were not analyzed. If, in fact, no platelet plugs are formed this is quite interesting in light of the fact that this is a common observation reported for hemorrhage induced by various other snake venom hemorrhagic toxins. Platelet plug formation is also indicative of endothelial cell damage. The absence of platelet plugs suggests that proteinase H exhibits disintegrin-like activity. This is a reasonable suggestion in light of the KINI and EVANS (1992) hypothesis that disintegrins and snake venom metalloproteinases are derived from a common precursor molecule. According to this hypothesis proteinase H, a high molecular weight metalloproteinase, would be expected to possess both a metalloproteolytic domain and a disintegrin-like domain. This expectation is supported by the absence of platelet plug formation following proteinase H administration *in vivo* and thus supports the Kini and Evans hypothesis. For this reason, future studies on proteinase H should include experiments of the inhibition of platelet aggregation.

In final summary, all three hypotheses of the dissertation were accepted. The proteolytic and hemorrhagic activities of proteinase H were dependent upon glycosylation. Proteinase H induced hemorrhage *per rhexis* secondary to microvascular endothelial cell cytotoxicity. Proteinase H was directly cytotoxic to microvascular endothelial cells in culture.

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Candidate for the Degree of

Doctor of Philosophy

Thesis: STUDIES ON THE HEMORRHAGIC TOXIN, PROTEINASE H, FROM *CROTALUS ADAMANTEUS* (EASTERN DIAMONDBACK RATTLESNAKE) VENOM

Major Field: Physiological Sciences

Biographical:

Personal Data: Born in Claremore, Oklahoma, February 28, 1969; the son of Gregory A. and Charlotte A. Anderson

Education: Received Bachelor of Science in Physiology from Oklahoma State University in May, 1991; completed requirements for Doctor of Philosophy in Physiological Sciences at Oklahoma State University in July, 1996.

Professional Experience: Research Assistant, Department of Physiological Sciences, Oklahoma State University, June 1996 to July 1996; Research Fellow, Department of Physiological Sciences, Oklahoma State University, September 1992 to May 1996; Teaching Assistant, Department of Physiological Sciences, Oklahoma State University, July 1991 to June 1992; Undergraduate Research Assistant, Department of Plant Biology, Oklahoma State University, 1989-1990.