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Peptidase activities in *Crotalus durissus terrificus* semen

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Abstract

To understand the role of peptidases in seminal physiology of *Crotalus durissus terrificus*, activity levels of representative enzymes in semen and their sensitivities to inhibitors, cofactors, and peptide hormones were evaluated. The existence of seminal fractions and the association of peptidases with these fractions were also characterized for the first time in snakes. The prominent inhibitors of aminopeptidases (APs) were amastatin for acid, basic, and neutral; bestatin for basic; and diprotin A for dipeptidyl-IV. Cystyl and prolyl-imino APs were similarly susceptible to the majority of these inhibitors. The basic and neutral were characterized as metallo-peptidases, acid AP was activated by $MnCl_2$, and cystyl, prolyl-imino, and type I pyroglutamyl were characterized as sulphydryl-dependent APs. Angiotensin II, vasotocin, bradykinin, fertilization-promoting peptide, and TRH altered the majority of these peptidase activities; these peptides are possible substrates and/or modulators of these peptidases. Peptidase activities were found in all seminal fractions: seminal plasma (SP), prostasome-like (PR) structures, and soluble (S-) and membrane-bound fractions (MFs) of spermatozoa. The levels of activity of each peptidase varied among different seminal fractions. In SP, the higher activities were puromycin-insensitive neutral and basic APs. In PR, the higher activity was puromycin-insensitive neutral AP. In spermatozoa, the higher activity in subcellular SF was puromycin-sensitive neutral, while in MF both puromycin-sensitive and -insensitive neutral APs were equally higher than the other examined peptidases. Data suggested that these peptidases, mainly basic and neutral, have a high relevance in regulating seminal functions of *C. d. terrificus*.

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Introduction

Enzyme hydrolysis leading to inactivation or processing of peptides has been assumed to be a limitation step for their biological activities. Many peptides, such as angiotensins (Angs; Fraser *et al*. 2006), kinins (Blaukat 2003), substance P, enkephalins (Sastry *et al*. 1991), endorphin (El-Haggar *et al*. 2006), oxytocin (OXT), vasopressin (AVP; Assinder *et al*. 2000), LHRH (Amory & Bremner 2003), TRH, and fertilization-promoting peptide (FPP; Green *et al*. 1996), as well some peptidases, particularly aminopeptidases (APs) and oligopeptidases have been related to seminal physiology in mammals (Fernández *et al*. 2002, Irazusta *et al*. 2004, Valdivia *et al*. 2004, Subirán *et al*. 2008). In this sense, Ang I and II are hydrolyzed by acid AP (APA; EC 3.4.11.7; Kugler 1982) and, also, by prolyl oligopeptidase (POP; EC 3.4.21.26; Barret *et al*. 1998). The formation of Ang IV from Ang III (Kugler 1982) and bradykinin (BK) from kallidin (Mizutani *et al*. 1993) is catalyzed by neutral AP (APN). Enkephalin is hydrolyzed by puromycin-insensitive APN (APN-PI; E.C 3.4.11.2) and puromycin-sensitive APN (APN-PS, E.C 3.4.11.14; Fernández *et al*. 2002). Substance P, enkephalin, BK, OXT, AVP, LHRH (Barret *et al*. 1998), TRH, and FPP (Siviter & Cockle 1995) are hydrolyzed by POP. OXT and AVP are hydrolyzed by cystyl AP (CAP, EC 3.4.11.3; Davison *et al*. 1993). LHRH and TRH are susceptible to type I pyroglutamyl AP (PAP-I, EC 3.4.19.3; Cummins & O'Connor 1998), and FPP is a possible substrate of this enzyme. BK, kallidin, met-enkephalin, and somatostatin are hydrolyzed by basic AP (APB, EC 3.4.11.6; Barret *et al*. 1998). Substance P and endorphin-2 are substrates of dipeptidyl peptidase-IV (DPPIV, EC 3.4.14.5; Barret *et al*. 1998).

The distribution of peptidases in seminal fractions is fundamental to the regulatory role of these enzymes. Until the present, earlier studies of human seminal fractions evaluated the distribution of only three of the above-mentioned peptidases: APN (Fernández *et al*. 2002, Subirán *et al*. 2008), PAP, and POP (Valdivia *et al*. 2004). APN was detected in soluble (S-) and solubilized membrane-bound fractions (MFs) of seminal plasma (SP) and prostasomes (PRs; Fernández *et al*. 2002). In sperm, APN was detected in the equatorial segment of the upper post-acrosomal region of the head, in the neck, and

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along the tail (Subirán *et al*. 2008). Alterations of APN levels in human semen were associated with different abnormalities in the sperm of subfertile patients (Irazusta *et al*. 2004). PAP-I and POP activities were detected in SP and in the PR fraction, as well as in soluble and membrane-bound sperm subcellular fractions, and have higher activities in necrozoospermia than in normozoospermic semen (Valdivia *et al*. 2004). AP and amylase activities are also known to be the main agents in the liquefaction process of the ejaculated semen coagulum, in humans (Chatterjee *et al*. 1997). Moreover, AP inhibitors, bestatin and puromycin, are capable of diminishing cellular proliferation and viability in mammals (Takahashi *et al*. 1989, Constam *et al*. 1995). However, bestatin is capable of restoring and promoting follicular growth and ovulation after its suppression by stress (Nakamura *et al*. 1998).

To our knowledge, the fractionation of semen and the presence of seminal APs and POP have not yet been studied in snakes. Furthermore, species possessing sperm with long lifespans, such as the rattlesnake *Crotalus durissus terrificus*, are interesting experimental models to study the distribution and function of peptidases in seminal fractions. In *C. d. terrificus*, spermatogenesis begins in spring and has its peak in summer. The spermatozoa are maintained in the male tract until mating (middle of autumn; Almeida-Santos *et al*. 2004) and, subsequently, in the female tract until ovulation (spring; Almeida-Santos & Salomão 1997). The goal of the present study was to evaluate the existence of mammalian-like seminal fractions (SP, PR, and sperm), and to characterize the activity levels of soluble and membrane-bound APA, APB, APN-PI, APN-PS, CAP, PAP-I, DPPIV, POP, and prolyl iminopeptidase (PIP) and their sensitivities to inhibitors, cofactors, and possible natural substrates in whole semen, as well as their associations with different seminal fractions of *C. d. terrificus*.

Results

Figure 1 shows that lactate dehydrogenase (LDH) activity in SF was threefold higher than in MF of whole semen. Similar proportion was obtained in spermatozoa subcellular SF and MF (data not shown).

Table 1 shows values of peptidase activity in SF and MF of whole semen. APB, PIP, and POP activities were detected only in SF, while the others were detected in SF and MF.

Table 2 shows that APB activity was markedly inhibited by amastatin and bestatin, APA and APN by amastatin, and diprotin A had a stronger effect on DPPIV activity in SF and MF. CAP and PIP activity decreased in the presence of majority of examined inhibitors. Bestatin and puromycin were more efficient to inhibit PAP. POP activity was not evaluated by classical AP inhibitors, since it is an endooligopeptidase (the only one under study here), and this activity was shown to be increased

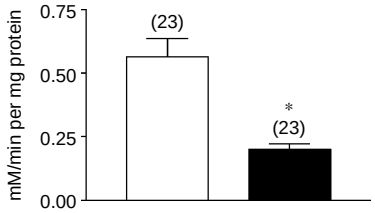

Figure 1 LDH activity (means±S.E.M.) in soluble (white bar) and solubilized membrane-bound (black bar) fractions of whole semen of *C. d. terrificus*. Number of animals is given in parentheses. *$P<0.0003$ (unpaired two-side Student's *t*-test).

or indifferent with the adopted agents (Olivo *et al*. 2008). CAP activity in SF, PAP-I, and PIP had a common pattern of decrease with BK, TRH, and FPP (with or without Ca^{2+}). Ang II and BK diminished APA activity in SF and MF. Ang II and arginine vasotocin (AVT) decreased CAP activity in SF and PIP activity. AVT also decreased CAP activity in MF. DL-dithiothreitol (DTT) improved CAP activity in SF and MF and PIP activity, and decreased APB activity. EDTA diminished the activities of APB and APN SF and MF. $MnCl_2$ decreased the activities of CAP in MF, PAP, and DPPIV SF and MF, and increased APA activity.

As shown in Fig. 2, a peak coincided with the dead volume (Vo) of seminal fraction from gel filtration in Sephadex G-200. This peak was analyzed by transmission electron microscopy and had numerous rounded or oval membranous vesicles of the size 50–200 nm, shown in Fig. 3.

Figure 4 shows AP activities in seminal fractions: SP, PR, and spermatozoa subcellular SF and MF. In SP, the higher activities were APN-PI and APB relative to other examined peptidases. In PR, APN-PI activity also had the higher level, followed by APB and APN-PS. In spermatozoa subcellular SF, APN-PS activity was higher, while in spermatozoa subcellular MF, APN-PS and APN-PI

Table 1 Activity levels of aminopeptidases: acid (APA), basic (APB), puromycin-insensitive neutral (APN-PI), puromycin-sensitive neutral (APN-PS), dipeptidyl peptidase-IV (DPPIV), pyroglutamyl-I (PAP-I), prolyl-imino (PIP); and prolyl oligopeptidase (POP) in soluble (SF) and solubilized membrane-bound (MF) fractions of whole semen of *Crotalus durissus terrificus*.

	Activities (U/mg protein)	
Aminopeptidase	SF	MF
APA	691 ± 180^{a} (16)	838 ± 229^{a} (16)
APB	$18\,549\pm3227^{b,*}$ (15)	Absent
APN-PI	$30\,372\pm3107^{c}$ (13)	$23\,866\pm1938^{b}$ (13)
APN-PS	$9019\pm1133^{d,*}$ (10)	$12\,637\pm1290^{c}$ (11)
CAP	31 ± 14^{a} (14)	30 ± 13^{a} (15)
DPPIV	$234\pm57^{a,*}$ (13)	560 ± 153^{a} (11)
PAP-I	261 ± 34^{a} (14)	231 ± 71^{a} (16)
PIP	$257\pm66^{a,*}$ (15)	Absent
POP	$1777\pm252^{a,d,*}$ (14)	Absent

Values are means±S.E.M. Number of animals is given in parentheses. U=picomoles of hydrolyzed substrate per minute. Different letters indicate different peptidase activity levels in the same fraction (One-way ANOVA $P<0.0001$, Bonferroni $P<0.05$). *$P<0.05$ in comparison to MF (unpaired, two-side Student's *t*-test).

Table 2 Remaining activities of aminopeptidases: acid (APA), basic (APB), puromycin-insensitive neutral (APN-PI), puromycin-sensitive neutral (APN-PS), cystyl (CAP), dipeptidyl peptidase-IV (DPPIV), pyroglutamyl-I (PAP-I), prolyl-imino (PIP); and prolyl oligopeptidase (POP) in soluble (SF) and solubilized membrane-bound (MF) fractions of whole semen of *Crotalus durissus terrificus* with classical aminopeptidase inhibitors and angiotensin II (AngII), arginine vasotocin (AVT), bradykinin (BK), fertilization-promoting peptide (FPP), fertilization-promoting peptide (FPP) plus calcium (FPP+Ca[2+]), TRH, DL-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA) and $MnCl_2$.

	Remaining peptidase activity (% control)											
	APA		APB	APN		CAP		DPPIV		PAP-I		PIP	POP
Agents	SF	MF		SF	MF	SF	MF	SF	MF	SF	MF		
Amastatin	11±1*	21±3*	5±2*	9±4*	14±2*	83±18	0±7*	51±7*	89±14	78±19	92±27	78±22	–
Bestatin	76±7*	33±7*	7±2*	69±9*	21±5*	8±2*	0±5*	52±9*	91±12	56±9*	45±12*	16±9*	–
Diprotin A	99±8	84±11	112±23	100±12	54±8*	48±5*	0±4*	51±9*	68±11*	97±21	92±24	67±5*	–
Puromycin	97±13	75±15	95±11	93±11	45±9*	34±7*	11±4*	69±11*	91±11	56±13*	61±9*	44±7*	–
AngII	40±10*	35±2*	105±18	104±8	84±12	20±9*	0±3*	89±11	85±19	94±11	82±16	29±7*	118±22
AVT	99±2	78±11	112±17	105±8	80±11	28±9*	0±6*	97±13	94±21	199±21	89±32	38±9*	140±32
BK	77±11*	65±9*	95±9	102±7	84±11	0±6*	0±5*	91±12	92±14	59±16*	34±18*	7±4*	140±20
FPP	81±9	98±16	99±8	105±18	88±10	36±11*	0±6*	85±15	91±14	0±12*	0±9*	46±6*	149±20
FPP+Ca[2+]	96±11	84±19	102±9	104±12	87±21	10±3*	0±9*	72±8*	82±18	17±8*	37±11*	15±2*	151±21
TRH	92±11	95±7	101±15	102±11	84±11	0±3*	0±6*	86±9	89±17	63±13*	41±19*	4±1*	161±24
DTT	86±9	71±19	42±10*	106±11	88±14	2510±193*	7587±654*	88±8	83±18	114±18	129±24	1302±211*	128±30
EDTA	88±9	92±6	39±9*	74±9*	16±3*	122±24	236±56	95±10	86±9	81±23	90±21	110±14	103±18
$MnCl_2$	356±42*	452±51*	86±14	107±8	70±17	84±18	0±2*	67±8*	73±4*	0±14*	0±16*	74±28	117±22

Values are means±S.E.M. from triplicates of pool of 11 animals. Control (absence of any factor) = 100%. *$P<0.05$ in comparison with the respective control (unpaired, two-side Student's *t*-test).

activities were higher than other examined peptidase activities. Considering activity levels of each peptidase among different seminal fractions, APB, PIP, and POP were higher in SP; APB, APN-PI, DPPIV, and PAP-I in PR; APN-PS in spermatozoa subcellular SF; and PAP-I and CAP in spermatozoa subcellular MF. APA was equally distributed among all seminal fractions. It was noteworthy that APN-PS activity was absent in SP.

Discussion

The efficiency of the employed procedure to separate SF and MF was confirmed by the pronounced LDH activity (cytosolic marker) in SF of all samples, which allowed the characterization of the activity of each peptidase in different fractions, and consequently the association of intra- and extracellular peptides with the peptidase activities under study. Overall, peptides involved in intracellular signaling are primarily hydrolyzed outside the cell, mainly by membrane-bound peptidases which have, in general, the active sites in the extracellular side, which interact with released peptides (O'Cuinn 1998). Soluble peptidases act mainly in intracellular processes, but can also be exocytosed or act in recaptured peptides, which are internalized as a part of the peptide-receptor complex, as occurs with peptide hormones (Gibson *et al.* 1989). Under stimulation, cytosolic enzymes can be translocated to the cell surface and efficiently process extracellular substances (Albiston *et al.* 2004). In the present study, APB, PIP, and POP activities were detected only in SF and the other activities were detected in SF and MF, following the same pattern found in tissues and semen of mammals (Irazusta *et al.* 2001, 2004, Silveira *et al.* 2001, Varona *et al.* 2003).

The use of substances derived from naphthylamide has been the initial step to detect or confirm the involvement of APs and POP in physiological mechanisms. Variables that could not be controlled might have influenced peptidase activities in the present study, e.g. intra-season and geographical and circadian variations. This is possible considering the seasonality, since samples were obtained in different days of the same season (austral autumn – the mating season for *C. d. terrificus*). However, the fertility potential of semen can be presumed effective and homogeneous along this season, based on the existence of a high quality of seminal dynamic parameters of *Boa constrictor occidentalis* (Tourmente *et al.* 2007), the peak of testosterone (Zacariotti *et al.* 2005) and the decrease of spermatozoa defects (Zacariotti 2004) in *C. d. terrificus*. Geographical variation was not a factor as snakes were captured from locations with similar fauna and environmental conditions. Moreover, we can also exclude circadian variations, because the material was obtained in the same period of the day, with a maximum difference of 90 min.

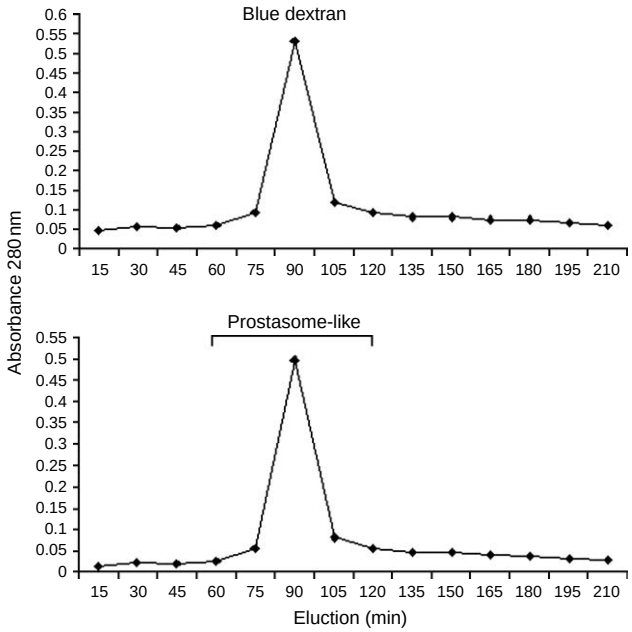

Figure 2 Gel filtration chromatography on Sephadex G-200. The column was equilibrated and washed with Tris–saline buffer (30 mM Tris plus 130 mM NaCl), pH 7.4, flow rate 0.16 ml/min.

The anatomy and morphology of the male reproductive tract could also influence the features of the peptidase activities and their distribution in seminal fractions of *C. d. terrificus*. In reptiles, the ductus deferens are recognized as the site of spermatozoa storage in males and, differently from mammals, the epididymis does not participate in sperm maturation and storage (Sever *et al*. 2002). The ductus deferens of *C. d. terrificus* are convoluted and enter the cloaca independently of the ureters. As previously described by Almeida-Santos *et al*. (2004), the ductus deferens occupy about one-third of the body length and when freed from the peritoneal membrane and manually distended, its length is about six times the total body length, although the right duct is always slightly longer than the left. Three regions can be distinguished: proximal (testicular), median, and distal (cloacal). In this snake, spermatozoa become increasingly more motile from the proximal to the distal region (Almeida-Santos *et al*. 2004). Regarding the presence of examined peptidase activities in the spermatozoa, the identification of their anatomical sources need to be investigated; for example, in the acrosomal complex, which is known in all vertebrate taxa as an enzyme-rich organelle that facilitates spermatozoal penetration into the ovum (Subirán *et al*. 2008). In squamates, the acrosomal complex is highly compartimentalized (Gribbins *et al*. 2007), and this compartimentalization has been suggested as an aid to the sequential release of acrosomal enzymes (Talbot 1991). Since the scope of the present study was not to compare the seminal fractions and peptidase composition from different areas of the ductus deferens, semen from the entire ductus deferens of *C. d. terrificus* was taken systematically to assure similar seminal fluid components for each analysis.

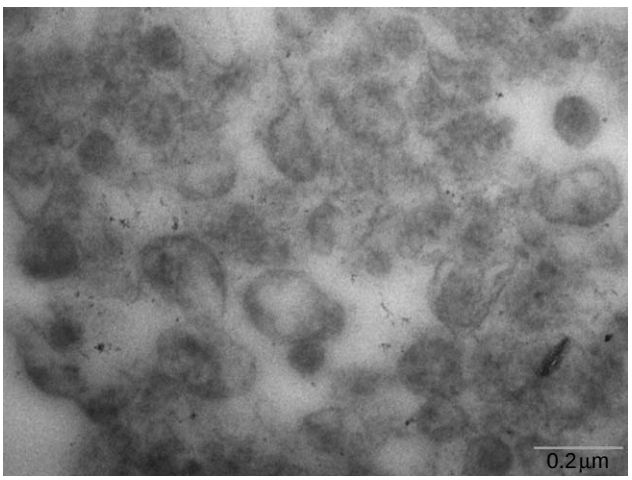

Figure 3 Electron microphotography of prostasome-like structures from semen collected from the ductus deferens of *C. d. terrificus* in austral autumn.

The APs under study are known to be sensitive to amastatin, bestatin, diprotin A, and puromycin (Cadel *et al*. 1995, Ronai *et al*. 1999, Sato 2003). POP is inhibited by Z-Pro-prolinal (García-Horsman *et al*. 2007) and belonging to a distinct class of enzymes, it was not included in the inhibition assay.

In the present study, APB susceptibility to bestatin distinguished this enzyme from APA and APN. By the susceptibility of DPPIV to diprotin A, and PAP-I, CAP, and PIP to puromycin, it was possible to differentiate them from APA and APB. The inhibition of DPPIV by diprotin A is a known feature of this enzyme (CD26) in mammals (Minelli *et al*. 1999). As distinguishing features, APB and APN are metallo-peptidases, because of their inhibition by EDTA (Kawata *et al*. 1980). APA was marked as activated by $MnCl_2$, which is a known characteristic of this enzyme in mammals (Ramírez *et al*. 1990). CAP and PAP-I were activated by DTT, a thiol-reducing agent, characterizing them as sulphydryl-dependent peptidases. DTT is an activator of the type I PAP and an inhibitor of the type II (O'Cuinn *et al*. 1990), and the degradation of FPP (possible substrate of PAP-I) is also increased by the presence of DTT (Cockle *et al*. 1994), which confirms that PAP-I is among the enzymes studied in the present work and FPP is one of its substrates. Overall, the present results show that seminal peptidase activities of *C. d. terrificus* have distinct biochemical properties, and therefore it is possible to attribute them to different proteins. It was notable that enzyme activities were affected by the evaluated peptides, which are their possible substrates (e.g. Ang II on APA, AVT on CAP, and PIP, and particularly TRH and FPP with or without Ca[2+] on CAP, PIP, and PAP-I). These results indicate the competition among these

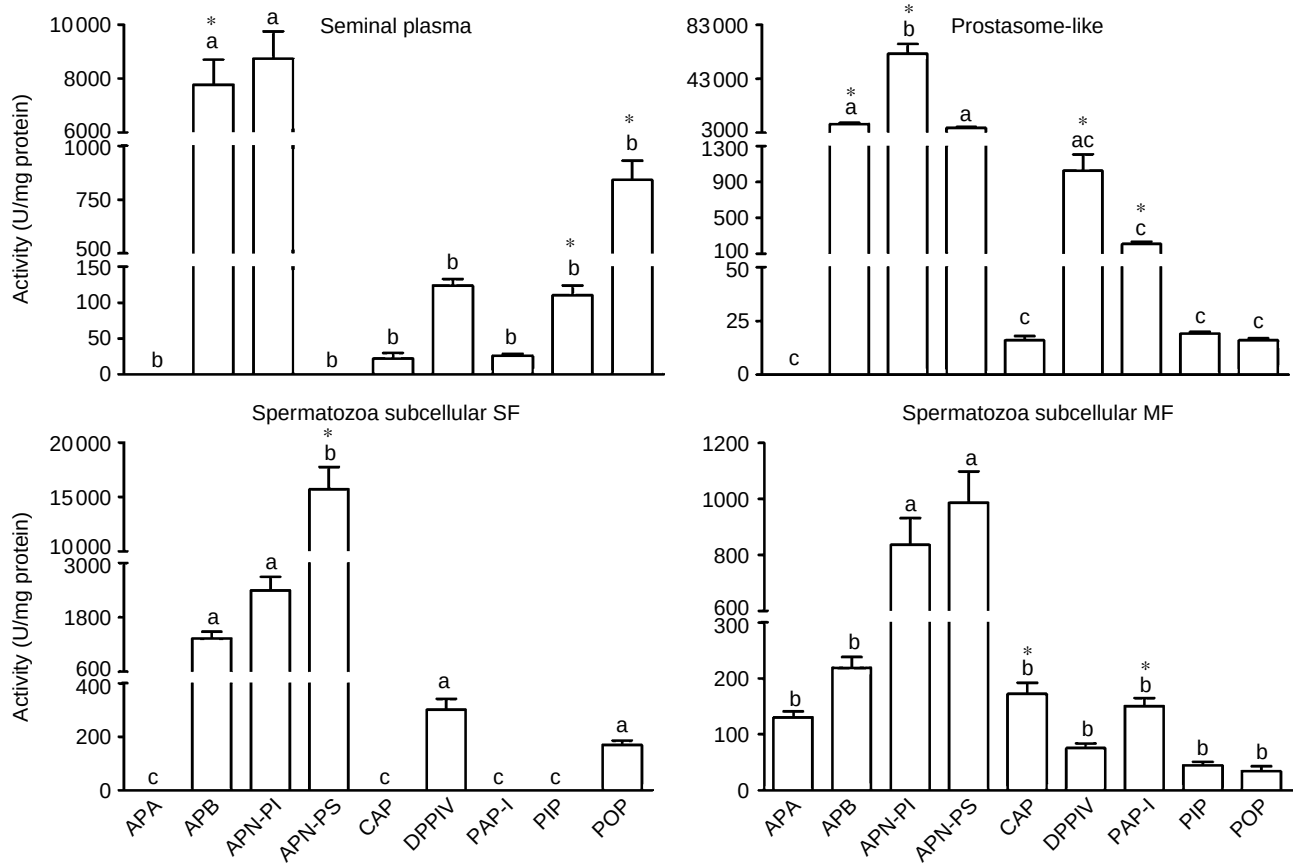

Figure 4 Distribution of activity levels of aminopeptidases: acid (APA), basic (APB), puromycin-insensitive neutral (APN-PI), puromycin-sensitive neutral (APN-PS), cystyl (CAP), dipeptidyl peptidase-IV (DPPIV), pyroglutamyl-I (PAP-I), prolyl-imino (PIP); and prolyl oligopeptidase (POP) in seminal plasma, prostasome-like and spermatozoa subcellular soluble (SF) and solubilized membrane-bound (MF) fractions of *C. d. terrificus*. Values are means ± S.E.M. from triplicates of samples (see Materials and Methods). U = picomoles of hydrolyzed substrate per min. Different letters indicate different peptidase activity levels in the same fraction (one-way ANOVA, $P<0.0001$, Bonferroni $P<0.001$). Asterisks indicate the higher activity of each peptidase when comparisons were made among all seminal fractions (one-way ANOVA, $P<0.0003$, Bonferroni $P<0.05$).

peptides and the synthetic substrates, strongly suggesting that those peptides are natural substrates of seminal peptidases of *C. d. terrificus*.

The semen of the rattlesnake contains SP and spermatozoa, as well as vesicles resembling mammalian PRs. Although ulterior ultrastructural analyses are needed to determine peculiarities of these vesicles seen in *C. d. terrificus*, compared with PRs of mammals, they could be considered as PR structures since they were obtained by the same purification procedure employed to obtain human PRs (Fernández *et al*. 2002, Vivacqua *et al*. 2004) and had peptidase activities (Fernández *et al*. 2002), shape (Fernández *et al*. 1997, Vivacqua *et al*. 2004), and size (Olsson & Ronquist 1990, Vivacqua *et al*. 2004) similar to human PRs. Mammalian PRs are membranous vesicles (50–550 nm; 150–200 nm are the most frequent), which contain large amounts of cholesterol, sphingomyelin, calcium, and several enzymes (Kravets *et al*. 2000). They are surrounded by a bilaminal or multilaminar lipoprotein membrane with unusual lipid composition due to quantitative predominance of cholesterol over phospholipids (Kravets *et al*. 2000). PRs have been related to sperm motility, liquefaction, and immunosuppression (Kravets *et al*. 2000). Since PRs can be identified only after purification procedures it is difficult to infer how frequent they are in *C. d. terrificus*. Another important question that remains to be investigated is the origin of these vesicles in *C. d. terrificus*. In humans, PRs are secreted by the prostate gland (Kravets *et al*. 2000). In bovines, PRs are originated in the seminal vesicles (Agrawal & Vanha-Perttulla 1987). Seminal vesicles secrete proteins, vitamin C, fructose, prostaglandin, and fibrinogen, while the prostate gland secretes a whitish liquid with low viscosity which contains citrate and Ca^{2+} (Minelli *et al*. 1998). It is noteworthy that *C. d. terrificus* possesses PR structures, since squamates do not possess a prostate gland, seminal vesicles, and bulbo-urethral and urethral glands (Sever 2004). The renal sexual segment is known to act as an accessory sex organ (Sever *et al*. 2002), from which those structures could have originated. This renal sexual segment of snakes secretes a complex of glycogen,

mucopolysaccharides, lipids, mucoproteins, and phosphatases (Kühnel & Krisch 1974) in a pattern that can be related to spermatogenic cycle and mating activity (Sever *et al*. 2002). However, since in the present study the semen was collected from the ductus deferens, it would not have mixed with fluids from the renal sexual segment but probably from other areas of major secretory activity in the snake urogenital system, e.g. the ampulla ductus deferentis and the ductuli epididymides of the epididymis (Volsoe 1944), from which the PR structures could have originated. The ductuli epididymides or ductuli efferents have been observed in the snake *Seminatrix pygaea* as efferent ducts from seminiferous tubules of the testis that lead into the epididymis, from which sperm pass into the ductus deferens (Sever 2004). In several mammals, the terminal portion of the ductus deferens is differentiated into an ampulla, which is considered a multifaceted organ, morphologically similar to the seminal vesicles (Riva *et al*. 1982). Among reptiles, the ampulla has been reported only in squamates, being more prominent in lizards than in snakes (Akbarsha *et al*. 2005). The ampulla of squamates and mammals shares the irregular narrow folded epithelium (Sever 2004).

The higher levels of the majority of the examined peptidase activities were found in SP and PR fractions. APN and APB activities were more expressive in all seminal fractions. The conversion of kallidin to BK (Mizutani *et al*. 1993) and inactivation of BK (Barret *et al*. 1998) are, respectively, actions of APN and APB, indicating the importance of the regulation of these peptides to seminal function in *C. d. terrificus*. However, considering our results and the large range of peptides that are susceptible to these peptidases, it is not possible to assure that the high levels of peptidases are restricted only to the kinin system. In other words, the relevance of other evaluated peptidases cannot be ignored. For instance, DPPIV and POP activities were also highly expressed in PR and SP of *C. d. terrificus* respectively. Considering their known hydrolytic effects over endorphin-2 and enkephalin, respectively (Barret *et al*. 1998), and the fact that endogenous opioid peptides seem to have a marked role in seminal physiology (O'Hara *et al*. 1994, Agirregoitia *et al*. 2006), DPPIV and POP activities may have a relevant role in seminal physiology of *C. d. terrificus*.

In conclusion, this is the first report of fractionation and peptidase composition of semen of reptiles, and demonstrated the presence of acid, basic, neutral (puromycin-sensitive and -insensitive), cystyl, dipeptidyl-IV, type I pyroglutamyl, and prolyl-imino APs, as well as POP activities in SP, spermatozoa, PR structures, and whole semen from ductus deferens of *C. d. terrificus*. Metallo-dependent APs inhibited by amastatin and bestatin (mainly those that act on neutral or basic amino acids) had marked levels in whole semen and in all seminal fractions, indicating a high physiological importance. Ang II, AVT, BK, FPP, and TRH altered the majority of the peptidase activities under study, suggesting them as possible substrates and/or modulators of these peptidases.

Materials and Methods

Animals

Adult male snakes (*C. d. terrificus*, Serpentes, Viperidae, Crotalinae; $n=26$) were captured from their natural environment in the states of São Paulo and Minas Gerais (Brazil) during austral autumn, when testosterone is at its peak (Zacariotti *et al*. 2005) and mating occurs (Almeida-Santos & Salomão 1997). The animals were identified by the Laboratory of Herpetology of the Instituto Butantan and housed individually in wooden cages (inside length×width×height of 35×26×22 cm) and acclimated to controlled conditions of temperature (25 °C), humidity (65.3±0.9%), and photoperiod (12 h light:12 h dark–lights on at 0600 h) in a restricted-access room for a period of 10 days.

After anesthesia with CO_2 exposure for 3 h, the ductus deferens of the reproductive tract were removed by laparotomy (Langlada *et al*. 1994), and semen was obtained from these ducts. The animals were then destined for other studies after euthanizing by decapitation.

The animal and research protocols used in this study are in agreement with the Brazilian Council Directive (COBEA-BRAZIL) and were approved by the Ethics Committee of the Instituto Butantan (193/04).

Collection of C. d. terrificus *semen*

As previously described by Almeida-Santos *et al*. (2004), the ductus deferens were stretched out on polystyrene plates for semen extraction. Pressure with a cell scraper (TPP – Techno Plastic Products AG, Trasadingen, Switzerland) along the whole extension of the two ducts was applied. The total amount of semen obtained was used for the following procedures.

Preparation of soluble and solubilized membrane-bound fractions from whole semen of C. d. terrificus

In order to obtain SF and MF, individual samples of whole semen were resuspended in 1 ml of 10 mM Tris–HCl buffer (pH 7.4), homogenized with a Teflon pestle in a glass potter (2 min at 800 r.p.m.) and ultracentrifuged (100 000 ***g*** for 35 min; Hitachi model HIMAC CP56GII). The resulting supernatants correspond to the SF of whole semen. To avoid contamination with the SF, the resulting pellet was washed three times with the same buffer and was then homogenized (2 min at 800 r.p.m.) in 1 ml of 10 mM Tris–HCl buffer plus 0.1% (v/v) Triton X-100 (Calbiochem, San Diego, CA, USA), and then ultracentrifuged (100 000 ***g*** for 35 min). The resulting supernatants correspond to the MF of whole semen. All steps were carried out at 4 °C. SF and MF were stored in polystyrene tubes at −80 °C until their use in LDH, protein, and peptidase activities assays.

Fractionation of semen of C. d. terrificus

In order to obtain SP, PR, and spermatozoa subcellular SF and MF of *C. d. terrificus* semen, the methodology described by Fernández *et al.* (2002) for human semen was adapted as follows. The pool of whole semen of five animals was homogenized in 1 ml Tris–saline buffer (30 mM Trizma (Sigma) plus 130 mM NaCl (Merck), pH 7.4) and centrifuged (600 ***g*** for 20 min; Hitachi HIMAC CP56GII). The supernatant contained SP and PR and was reserved for later use, while the pellet contained spermatozoa. To avoid contamination with SP and PR, the pellet was washed with 1 ml Tris–saline buffer and subsequently resuspended in 2.5 ml of the same buffer and centrifuged (600 ***g*** for 10 min). The resulting supernatant was discarded and the pellet was washed with 2.5 ml Tris–saline buffer and subsequently resuspended in 2.5 ml of the same buffer and centrifuged (1000 ***g*** for 15 min). The supernatant was discarded, and the resulting pellet contained only spermatozoa. This pellet was homogenized in 2.5 ml 10 mM Tris–HCl buffer (pH 7.4; 2 min at 800 r.p.m.) and stored at −80 °C, until use for separation of subcellular SF and MF. Then, the homogenate was sonicated (6 pulses of 30 s, with intervals of 15 s) and ultracentrifuged (100 000 ***g*** for 35 min). The pellet was reserved and the supernatant was ultracentrifuged (100 000 ***g*** for 35 min), and the resulting pellet was discarded while the supernatant was considered the spermatozoa subcellular SF. The reserved pellet from the penultimate ultracentrifugation was, then, homogenized at 800 r.p.m. (2 min, in 1 ml 10 mM Tris–HCl buffer) and ultracentrifuged (100 000 ***g*** for 35 min). After discarding the resulting supernatant, the pellet was homogenized at 800 r.p.m. (2 min, in 1 ml 10 mM Tris–HCl buffer), and was considered as the spermatozoa subcellular MF.

The resulting supernatant from the first centrifugation (SP and PR) was centrifuged (1000 ***g*** for 20 min) in order to eliminate debris and residual spermatozoa. The resulting pellet was discarded and the supernatant was stored at −80 °C. After defrost, this supernatant was ultracentrifuged (100 000 ***g*** for 120 min). The obtained pellet was reserved and the supernatant was ultracentrifuged at the same conditions. The resulting pellet was discarded and the resulting supernatant contained SP. The reserved pellet from the penultimate ultracentrifugation was washed with 0.6 ml Tris–saline buffer and subsequently resuspended in the same volume. To obtain 0.6 ml more of this resuspension, all needful procedures were repeated with another pool of whole semen of five animals. Both the resuspensions were mixed (1.2 ml), filtered in a membrane filter with a pore size 0.22 μm (Millipore, Bedford, MA, USA), and then submitted to gel filtration in Sephadex G-200 (Sigma; 1.5×30 cm), at a flow rate of 0.16 ml/min, in a column pre-equilibrated with Tris–saline buffer. The dead volume (Vo) was determined by injecting a solution of Blue dextran (Sigma). PR was not retained by the column (Fernández *et al.* 2002) and, then, was collected in the Vo (fractions 60–120 min or 9.6–19.2 ml), in a volume of about 9.6 ml. The eluate with PR was ultracentrifuged (100 000 ***g*** for 120 min) and the resultant pellet (PR fraction) was homogenized in 1 ml Tris–saline buffer. All procedures were performed at 4 °C. After obtaining SP, PR, SF, and MF of spermatozoa, samples were transferred to polystyrene tubes and maintained at −80 °C until the measurements of protein and peptidase activities. Part of PR fraction was destined to the electron microscopy.

Electron microscopy of PR fraction

The PR pellet was fixed for 2 h in a solution of 1.5% glutaraldehyde (Electron Microscopy Sciences-EMS, Hatfield, PA, USA) and 1% paraformaldehyde (Sigma) in 0.1 M cacodylate buffer, pH 7.3, and post-fixed in 1% osmium tetroxide in the same buffer. After dehydration in ethanol graded series, the pellet was embedded in Embed-812 resin (Electron Microscopy Sciences-EMS). Silver ultrathin sections were stained with uranyl acetate and lead citrate, examined and microphotographed (LEO 906E Transmission Electron Microscope, Zeiss, Göttingen, Germany).

Protein

Protein content was measured at 630 nm in triplicates by the method of Bradford (1976; Bio-Rad protein assay). Protein contents were extrapolated by comparison with standard curves of BSA (Sigma) in the same diluent.

LDH

As a marker for the fractionation procedure, LDH activity was determined (Bergmeyer & Brent 1972) in samples of 3 μl of SF and MF in triplicates incubated with 297 μl of NADH (β-NADH, reduced form; Sigma), dissolved in 100 mM phosphate buffer, pH 7.4, containing 1.6 mM sodium pyruvate (Sigma) and 200 mM NaCl. Absorbance was read in 96-well flat bottom microplates, at 0 and 10 min, in Bio-Tek PowerWave X spectrophotometer absorbance reader at 340 nm. Values of LDH activity were obtained by subtracting the absorbance reading at 10 min from time zero of incubation at 37 °C, and extrapolated by comparison with a standard curve of NADH dissolved in 100 mM phosphate buffer, pH 7.4, containing 200 mM NaCl. LDH activity was expressed as mmol NADH oxidized/min/mg protein.

Peptidase activity assays

Peptidase activities were quantified on the basis of the amount of 4-methoxy-β-naphthylamine (Sigma; for DPPIV and CAP) or β-naphthylamine (Sigma; for all other peptidases) released (Irazusta *et al.* 2001, Silveira *et al.* 2001, Gasparello-Clemente & Silveira 2002, Gasparello-Clemente *et al.* 2003, Varona *et al.* 2003), which is the result of the enzyme activity of 20–50 μl samples incubated with prewarmed substrate solution at concentrations of 0.125 mM (APA, APN-PS, APN-PI, CAP, PAP, PIP, and POP), 0.2 mM (DPPIV), and 0.5 mM (APB) in respective 0.05 M buffers containing BSA 0.1 mg/ml in 96-well flat bottom microplates for 30 min at 37 °C in a total volume of 300 μl. The content of naphthylamine was estimated fluorometrically (microplate fluorescence reader Bio-Tek FL600FA) at 460/40 nm emission wavelength and 360/40 nm excitation wavelength. The fluorescence value obtained at time zero was subtracted from values at time 30 min and the relative fluorescence was then converted to picomoles of β-naphthylamine (Sigma) or 4-methoxy-β-naphthylamine (Sigma) by comparison with a correspondent standard curve, which was dissolved in the same diluent as the incubation.

Peptidase activities were expressed as picomoles of hydrolyzed substrate per min (U) per milligram of protein, in which

the existence of a linear relationship between time of hydrolysis and protein content in the fluorometric assay was a previous condition. Considering enzyme activity measures as a comparative tool, the possible unspecific degradation during homogenization was not considered.

The following substrates and conditions were used:

- APA, L-aspartic acid α-(β-naphthylamide; Sigma; solubilized in 0.012 M NaOH) in Tris–HCl buffer, pH 7.4, with 1 mM $MnCl_2$;
- APB, L-arginine β-naphthylamide (Sigma; solubilized in H_2O) in phosphate buffer, pH 6.5, with 150 mM NaCl, and 0.02 mM puromycin (Sigma);
- APN, L-alanine-β-naphthylamide (Sigma; solubilized in 0.012 M HCl) in phosphate buffer, pH 7.4, with 1 mM DTT (Sigma), with or without puromycin – *APN-PI* activity is the result of the incubation with puromycin, while *APN-PS* is the result of values of incubates without puromycin minus those with puromycin;
- CAP, H-Cys-4-methoxy-β-naphthylamide (Bachem Bioscience Inc., Torrance, CA, USA; solubilized in 0.012 M HCl) Tris-maleate, pH 5.9;
- DPPIV, Glycil-Proline-4-methoxy-β-naphthylamide (Bachem Bioscience Inc.; solubilized in dimethyl sulfoxide, DMSO (Sigma)) in Tris–HCl buffer, pH 8.3;
- PAP-I, L-pyroglutamic acid-β-naphthylamide (Sigma; solubilized in DMSO) in phosphate buffer, pH 7.4, with 2 mM DTT (DTT inhibits PAP-II and actives PAP-I (O'Cuinn *et al.* 1990)) and 2 mM EDTA (Merck);
- PIP, L-proline-β-naphthylamide (Sigma; solubilized in DMSO) in phosphate buffer, pH 7.4;
- POP, Z-Gly-Pro-β-naphthylamide (Bachem Bioscience Inc.; solubilized in DMSO) in phosphate buffer, pH 7.4, with 2 mM DTT.

Characterization of peptidase activities in SF and MF of whole semen

The characteristics of peptidase activities with their classical inhibitors

The susceptibilities of APA, APB, APN, CAP, DPPIV, PAP-I, and PIP peptidase activities were comparatively evaluated in a SF and/or MF pool of whole semen from 11 animals with: amastatin ([(2S,3R)-3-Amino-2- -5-methylhexanoyl]-Val-Val-Asp-OH.HCl; Bachem Bioscience Inc.): 0.026 mM in acetic acid 8%; bestatin ([(2S,3R)-3-Amino-2-hydroxy-4- phenylbutanoyl]-L-leucine; Bachem Bioscience Inc.): 0.026 mM in acetic acid 8%; diprotin A (H-Lle-Pro-Lle-OH; Bachem Bioscience Inc.): 0.026 mM in deionized water; puromycin (3′-[α-Amino-*p*-methoxyhydrocinnamamido]-3-deoxy-*N*,*N*-dimethyladenosine): 0.026 mM in deionized water. The samples were incubated in the presence or absence of those substances, for 30 min, at 25 °C, and peptidase activities were quantified.

The characteristics of peptidase activities with peptide hormones, DTT, EDTA, and $MnCl_2$

The susceptibilities of APA, APB, APN, CAP, DPPIV, PAP-I, and PIP peptidase activities were comparatively evaluated in a SF and/or MF pool of whole semen from 11 animals with: 0.315 μM Ang II (Sigma); 0.314 μM AVT (reptilian analog of OXT and AVP; Sigma); 0.310 μM BK (Sigma); 0.94 μM FPP (Sigma); 0.94 μM FPP +1.8 mM $CaCl_2$; 0.911 μM TRH (Sigma); 0.97 mM DTT; 2 mM EDTA; 1 mM $MnCl_2$. The samples were incubated in the presence or absence of these substances, for 30 min, at 25 °C, and peptidase activities were subsequently quantified.

Statistical analysis

Data were analyzed statistically using GraphPad Prism and Instat softwares (GraphPad Software Inc., San Diego, CA, USA). Regression analysis was performed to obtain standard curves. ANOVA, followed by Bonferroni test, compared values among all peptidase activities in the same seminal fraction. Student's *t*-test was performed to compare values of LDH between SF and MF, and peptidase activities in different incubation conditions relative to the respective controls. Differences were considered statistically significant at a minimum level of $P<0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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