J Comp Physiol B (2009) 179:635–642
DOI 10.1007/s00360-009-0345-5

ORIGINAL PAPER

Peptidase activities in the semen from the ductus deferens and uterus of the neotropical rattlesnake *Crotalus durissus terrificus*

Camila Eduardo Marinho · Selma Maria Almeida Santos · Simone Cristina Yamasaki · Paulo Flavio Silveira

Received: 20 June 2008 / Revised: 4 November 2008 / Accepted: 31 January 2009 / Published online: 19 February 2009

Abstract To understand the role of peptidases in seminal physiology of *Crotalus durissus terrificus*, intra- and inter-seasonal activity levels of acid (APA), basic (APB), puromycin-sensitive (APN-PS) and puromycin-insensitive neutral (APN-PI), cystyl (CAP), dipeptidyl-IV (DPPIV), type-1 pyroglutamyl (PAP-I) and prolyl-imino (PIP) aminopeptidases as well as prolyl endopeptidase (POP) were evaluated in soluble (SF) and/or membrane-bound (MF) fractions of semen collected from the ductus deferens of the male reproductive tract and from the posterior portion of the uterus. Seminal APB, PIP and POP were detected in SF, while other peptidases were detected in SF and MF. Only the convoluted posterior uterus in winter and autumn had semen. Relative to other examined peptidases, in general, APN-PI, APN-PS and APB activities were predominant in the semen from the uterus and throughout the year in the semen from the ductus deferens, suggesting their great relevance in the seminal physiology of *C. d. terrificus*. The levels of peptidase activities in the ductus deferens semen varied seasonally and were different from those of semen in the uterus, suggesting that their modulatory actions on susceptible peptides are integrated to the male reproductive cycle events and spermatozoa viability of this snake.

Keywords Peptidases · Semen · Ductus deferens · Uterus · Reptile

Communicated by I. D. Hume.

C. E. Marinho · S. C. Yamasaki · P. F. Silveira (✉)
Laboratory of Pharmacology, Instituto Butantan,
Av. Vital Brasil, 1500, São Paulo, SP 05503-900, Brazil
e-mail: pefesil@butantan.gov.br

S. M. Almeida Santos
Laboratory of Ecology and Evolution, Instituto Butantan,
Av. Vital Brasil, 1500, São Paulo, SP 05503-900, Brazil

C. E. Marinho
Department of Physiology, Instituto de Biociências,
Universidade de São Paulo, São Paulo, SP 05508-900, Brazil

Introduction

Many peptides, such as angiotensins (Angs) (Fraser et al. 2001; Mededovic and Fraser 2005; Vinson et al. 1996), kinins (Schill et al. 1989), substance P (Sastry et al. 1991), enkephalins (O'Hara et al. 1994), endorphin (El-Haggar et al. 2006), oxytocin (OXT), vasopressin (AVP) (Assinder et al. 2002), luteinizing hormone-releasing hormone (LHRH) (Amory and Bremner 2003), thyrotropin-releasing hormone (TRH) and fertilization promoting peptide (FPP) (Green et al. 1996; Fraser et al. 2001) as well as related peptidases (Chatterjee et al. 1997; Fernández et al. 2002; Irazusta et al. 2004; Subirán et al. 2008; Valdivia et al. 2004) have been implicated in seminal functions in mammals. Ang II increases sperm motility, hyperactivation and capacitation (Vinson et al. 1996) without inhibition of spontaneous acrosome loss (Mededovic and Fraser 2005). Kinins enhance motility of ejaculated sperm (Schill et al. 1989). Substance P increases sperm motility (Sastry et al. 1991). Enkephalins are involved in spermatogenesis and reduced sperm motility (O'Hara et al. 1994). OXT and analogs induce contraction of seminiferous tubules and spermiation in rats (Assinder et al. 2002). FPP and TRH stimulate the capacitation and fertilizing ability of incapacitated sperm, but halt capacitation in capacitated sperm by inhibiting spontaneous acrosome loss (Fraser et al. 2001). Ang I and II are known to be hydrolyzed by acid aminopeptidase (APA) (EC 3.4.11.7) (Kugler 1982) and prolyl

endopeptidase (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 aminopeptidase (APN). Enkephalin is hydrolyzed by puromycin-sensitive APN (APN-PS, EC 3.4.11.14) and puromycin-insensitive APN (APN-PI, EC 3.4.11.2) (Fernández et al. 2002). Substance P, enkephalin, BK, OXT, AVP, LHRH (Barret et al. 1998), TRH and FPP (Siviter and Cockle 1995) are hydrolyzed by POP. OXT and AVP are also hydrolyzed by cystyl aminopeptidase (CAP, EC 3.4.11.3) (Davison et al. 1993) and potential substrates of proline iminopeptidase (PIP, EC 3.4.11.5) (Himmelhoch 1970). LHRH, TRH and FPP are substrates of type-1 pyroglutamyl aminopeptidase (PAP-I, EC 3.4.19.3) (O'Cuinn et al. 1990). BK, kallidin, met-enkephalin and somatostatin are hydrolyzed by basic aminopeptidase (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). Aminopeptidase and amylase activities are also known to promote liquefaction of the ejaculated semen coagulum in humans (Chatterjee et al. 1997).

All factors involved in spermatozoa survival are still unknown. Species possessing sperm with long life spans can be interesting experimental models to study seminal physiology, including the role of peptidases. In the rattlesnake *Crotalus durissus terrificus*, spermatogenesis begins in austral spring and has its peak in austral summer (Salomão and Almeida-Santos 2002). The spermatozoa are reported to be maintained in the ductus deferens until mating (middle of austral autumn) and subsequently in the posterior region of the oviduct (posterior uterus) until the ovulation and fertilization (austral spring) (Almeida-Santos and Salomão 1997; Almeida-Santos et al. 2004b). Consequently, ejaculated spermatozoa have been considered as maintained in the female reproductive tract, without loss of their viability, during the period of storage (austral winter) (Almeida-Santos and Salomão 1997). The number of spermatozoa of *C. d. terrificus* is increased in austral summer and autumn (which is related to mating during the middle of austral autumn) compared to austral winter (post-mating) (Almeida-Santos et al. 2004a). The survival time of spermatozoa of *C. d. terrificus* exposed to room temperature (20–24°C) was about 8 h and spermatozoa collected from snakes kept at −10°C for 24 h after death still had motility when defrost (Almeida-Santos et al. 2004a), suggesting that under these conditions components from the semen itself preserve the viability of spermatozoa. Concerning the semen in male snakes, the ductus deferens are recognized as the site of storage (Almeida-Santos et al. 2004a) and, differently from mammals, the epididymis does not participate in spermatozoa maturation and storage (Sever et al. 2002). However, controversial data exist about the storage of viable semen in female snakes. Although semen storage in the posterior uterus needs to be confirmed by histochemical and ultrastructural analysis from the entire uterus throughout the entire reproductive season, some reports show evidences that *C. d. terrificus* has evolved storage of viable semen in the posterior uterus, e.g. this uterine region becomes convoluted and contracted soon after mating, motile spermatozoa are found in this region in the winter, the twisting and convolution of this region could result in an obstruction which inhibits the passage of spermatozoa to the oviduct until ovulation and the subsequent fertilization, as well as endocrine factors seem to synchronize uterine relaxation with the ovulation (Almeida-Santos and Salomão 1997; Almeida-Santos et al. 2004b; Yamanouye et al. 2004). Nilson and Andrén (1982) hypothesized that secretions from the renal sexual segment caused this uterine contraction in *Vipera berus*. On the contrary, the existence of semen in the posterior uterus of pit vipers has been suggested as an artifact of mating activity, e.g. semen in the posterior uterus of *Agkistrodon piscivorus* has been reported to undergo degradation shortly before ovulation and the histology of the mid and anterior oviduct revealed that semen was actually migrating from the posterior uterus to infundibular tubules in this snake (Siegel and Sever 2008).

To highlight the involvement of APA, APB, APN-PS, APN-PI, CAP, DPPIV, PAP-I, PIP and POP in seminal physiology and in the ability to maintain the survival of spermatozoa of *C. d. terrificus*, the present study evaluated intra- and inter-seasonal levels of these peptidases in soluble fraction (SF) and solubilized membrane-bound fraction (MF) from semen collected from the entire ductus deferens as well as compared these activities between semen collected from the entire ductus deferens and semen collected from the posterior portion of the uterus of *C. d. terrificus*.

Materials and methods

Animals

The utilization of snakes was approved by the Ethics Committee of the Instituto Butantan (CEUAIB), protocol 193/04, in agreement with the ethical principles for experiments on animals of the Brazilian Council Directive (COBEA). Adult snakes (*C. d. terrificus*, Serpentes, Viperidae, Crotalinae) were captured from their natural environment in the states of São Paulo and Minas Gerais, and identified by the Laboratory of Herpetology of the Instituto Butantan. These snakes were housed individually in wood cages (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 6:00 a.m.) in a restricted-access room for a period of 10 days.

In all procedures, snakes were anesthetized with CO_2 exposure for 3 h. After ventral dissection, the reproductive tract was examined macroscopically.

Collection of semen

The ductus deferens of the male reproductive tract and the posterior portion of the uterus were removed by laparotomy (Langlada et al. 1994). The tissues were stretched in polystyrene plates and a gentle pressure with a cell scraper (TPP, Techno Plastic Products AG, Switzerland) along their whole extension was used to withdraw the semen. To assure similar components, semen from the entire ductus deferens was collected (Almeida-Santos et al. 2004a). Semen can be found in the ductus deferens throughout the year. With regard to the semen collected from the posterior portion of uterus, from the total number of 43 non-pregnant snakes examined, a subsample of 8 had semen in the uterus, 6 in winter and 2 in autumn. Thus, considering only the females collected in winter ($n = 11$), 54.54% had semen in the uterus. All uteri that had semen were also convoluted and contracted as described by Almeida-Santos and Salomão (1997). Since half of the examined female snakes had semen in the uterus in winter, mating occurred in 50% of the females, a finding that corroborates the existence of a biennial reproductive cycle in *C. d. terrificus*. However, mating could not have occurred in the year we studied these females with semen in the uterus, i.e. semen storage over more than 1 year should not be excluded. The other half of examined females that had no semen in the uterus could be in primary vitellogenesis, which reinforces the existence of a biennial cycle, or they may not have found a partner. Only semen samples collected from the uterus during winter were used in the present study. All semen samples possessed an aspect resembling a paste with gel-like viscosity, color varied from white to ivory and non-liquefaction within 120 min. The existence of motile spermatozoa was confirmed by optical microscopy in a drop of all semen samples. After laparotomy, the animals were killed by decapitation and destined for other studies. Variables that could not be controlled might have influenced peptidase activities in the present study, e.g. seminal contamination, geographic and circadian variations. This is possible considering seminal contamination, since samples were obtained by squeezing the ductus deferens and the posterior portion of the uterus. Although carefully performed, this procedure does not insure that the semen is free of duct or uterine cells, mucus, other fluids, etc. In any case, examined soluble and solubilized membrane-bound fractions represented, respectively, major soluble and membranal seminal elements plus minor contaminants. Due to these limitations, some differences could not be revealed in the present study. Furthermore, the scope of the present study was not to compare isolated spermatozoa, but whole seminal peptidase composition. Different secretions probably contribute to the composition of semen from the ductus deferens and the uterus of snakes. The renal sexual segment, a kidney specialization found only in males, secrets material into the collecting ducts and eventually into the cloaca where it mixes with seminal elements (Bishop 1959). Regarding the geographic variations, they do not seem to be a relevant factor, as snakes were provided from locations where fauna and weather were similar. Moreover, we can also exclude the circadian variations, because the material was obtained in the same period of the day, with a maximum difference of 90 min.

Preparation of soluble and solubilized membrane-bound fractions

In order to obtain soluble (SF) and solubilized membrane-bound (MF) fractions, individual samples (0.6–0.8 mL) of semen were added to 1 mL of 10 mM Tris–HCl buffer (pH 7.4), homogenized with a Teflon pestle in a glass potter (2 min at 800 rpm) and ultracentrifuged (100,000×*g* for 35 min) (Hitachi model HIMAC CP56GII). The resulting supernatants corresponded to the SF. 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 rpm) in 10 mM Tris–HCl buffer plus 0.1% Triton X-100 (Calbiochem, USA), and then ultracentrifuged (100,000×*g* for 35 min). The resulting supernatants corresponded to the MF of whole semen. All steps were carried out at 4°C. After the fractionation procedure, SF and MF were transferred to polystyrene tubes and stored at −80°C until the lactate dehydrogenase (LDH), protein and peptidase activities assays.

Lactate dehydrogenase

As a marker for the fractionation procedure, LDH activity was determined (Bergmeyer and Brent 1972) in samples of 3 µL of SF and MF in triplicates with 297 µL of NADH (*β*-nicotinamide adenine dinucleotide, reduced form) (Sigma, USA), dissolved in 100 mM phosphate buffer (pH 7.4) containing 1.6 mM pyruvate (Sigma, USA) and 200 mM NaCl. LDH assay was performed in 96-well microplates (Corning Co., USA) at 340 nm at 0 and 10 min in the Bio-Tek Power Wave X® spectrophotometer absorbance reader. Values of absorbance read at 10 min were subtracted from time 0 and extrapolated by comparison with a standard curve of NADH dissolved in 100 mM phosphate buffer containing 200 mM NaCl. LDH activity was expressed as mmoles of NADH oxidized per minute per milligram of protein.

Protein

Protein content was measured at 630 nm in triplicates using a Bio-Rad protein assay kit (Bio-Rad Laboratories, USA) (Bradford 1976), in the Bio-Tek Power Wave X® spectrophotometer absorbance reader. Protein contents were extrapolated by comparison with standard curves of bovine serum albumin (BSA) in the same diluent as the samples.

Peptidase activities

Peptidase activities were quantified on the basis of the amount of 4-methoxy-β-naphthylamine (for DPPIV and CAP) or β-naphthylamine (for all other peptidases) released (Gasparello-Clemente et al. 2003; Irazusta et al. 2004). This release is the result of the incubation in 96-well flat bottom microplates (for 30 min, at 37°C) of 20–50 µL SF and MF of whole semen 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 a total volume of 300 µL. The content of methoxy-β-naphthylamine (Sigma, USA) and β-naphthylamine (Sigma, USA) 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 0 (blank) was subtracted and the relative fluorescence was then converted to picomoles of methoxy-β-naphthylamine or β-naphthylamine 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 minute per milligram of protein. The existence of a linear relation 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, USA) (solubilized in 0.012 N NaOH) in Tris–HCl buffer (pH 7.4) with 1 mM $MnCl_2$;
- *APB* L-arginine β-naphthylamide (Sigma, USA) (solubilized in H_2O) in phosphate buffer (pH 6.5) with 150 mM NaCl, and 0.02 mM puromycin (Sigma, USA);
- *APN* L-alanine-β-naphthylamide (Sigma, USA) (solubilized in 0.012 N HCl) in phosphate buffer (pH 7.4) with 1 mM DL-dithiothreitol (DTT) (Sigma, USA), with or without 0.02 mM puromycin; *APN-PI* activity results 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., USA) (solubilized in 0.012 N HCl) in Tris–maleate (pH 5.9);
- *DPPIV* glycil-L-proline-4-methoxy-β-naphthylamide (Bachem Bioscience Inc., USA) (solubilized in dimethyl sulfoxide, DMSO, Sigma, USA) in Tris–HCl buffer (pH 8.3);
- *PAP-I* L-pyroglutamic acid-β-naphthylamide (Sigma, USA) (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 ethylenediaminetetraacetic acid (EDTA) (Merck, Brazil);
- *PIP* L-proline-β-naphthylamide (Sigma, USA) (solubilized in DMSO) in phosphate buffer (pH 7.4);
- *POP* Z-Gly-Pro-β-naphthylamide (Bachem Bioscience Inc., USA) (solubilized in DMSO) in phosphate buffer (pH 7.4) with 2 mM DTT.

Statistical analysis

Data were analyzed statistically using GraphPad Prism™ and Instat™ softwares. Regression analysis was performed to obtain standard curves of NADH, protein, β-naphthylamine and 4-methoxy-β-naphthylamine. Analysis of variance (ANOVA), followed by the Bonferroni test, compared values of each peptidase activity of semen during seasons, and among all peptidase activities in the same season. Student's *t* test was performed to compare paired values of LDH of SF and MF from the same sample, and peptidase activities in SF and MF of semen collected from male and female. All values were considered statistically significant at a level lower than 0.05.

Results

Lactate dehydrogenase

Lactate dehydrogenase activity was always higher in SF than in MF (about sixfold higher in semen collected from the uterus and threefold higher in semen from ductus deferens), indicating the efficiency of the fractionation procedure (data not shown).

Peptidase activities in the whole semen collected from the ductus deferens or uterus

Figure 1 shows comparisons of intra-seasonal levels of different peptidase activities and inter-seasonal levels of each peptidase activity of semen collected from the ductus deferens and uterus. In general, independently of seasonal fluctuation, the detectable peptidase activities were APB, PIP and POP in SF, and APA, APN-PI,

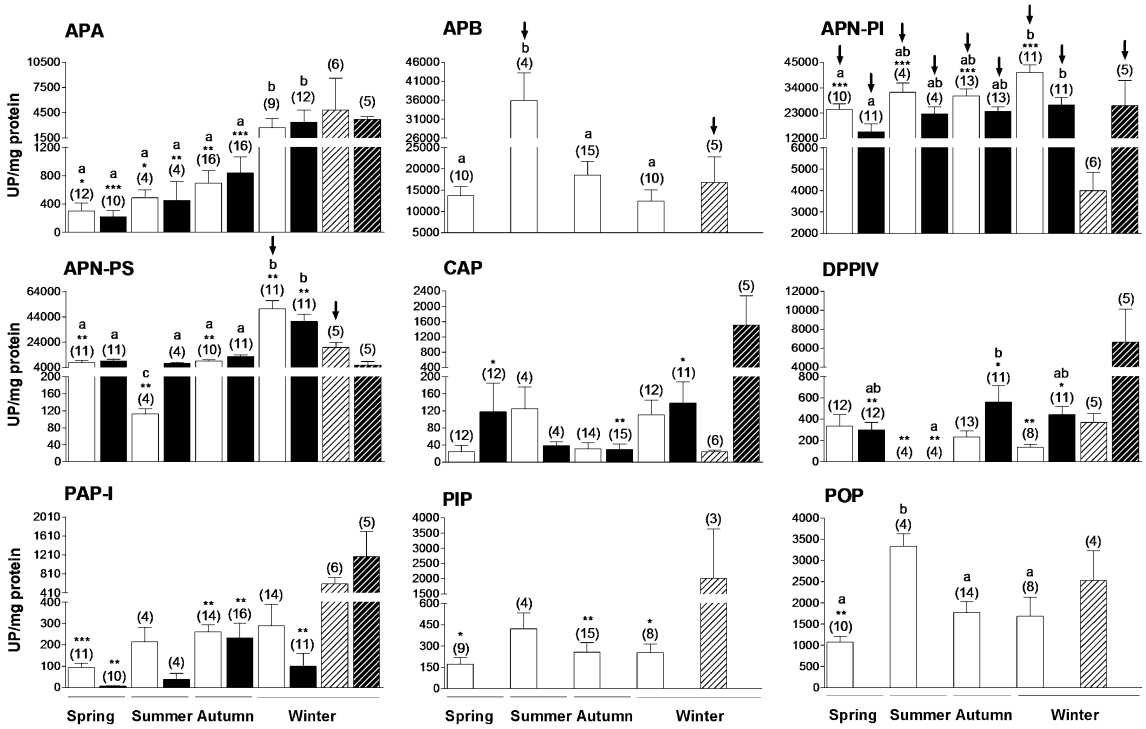

Fig. 1 Comparisons of activity levels (picomoles of hydrolyzed substrate per minute per milligram of protein, mean ± SEM) of acid (APA), basic (APB), puromycin-insensitive (APN-PI) and -sensitive neutral (APN-PS), cystyl (CAP), dipeptidyl-IV (DPPIV), type-1 pyroglutamyl (PAP-I) aminopeptidases, prolyl iminopeptidase (PIP) and prolyl endopeptidase (POP) in soluble (*white-plain* and *white-striped bars*) and solubilized membrane-bound fraction (*black-plain* and *black-striped bars*) of semen collected from the ductus deferens (*plain bars*) and from the uterus (*striped bars*) of *C. d. terrificus* during the wet season (austral spring/summer) and dry season (austral autumn/winter). *Arrows* indicate the highest activity levels of the same fraction of semen collected from the ductus deferens or collected from the uterus among all examined peptidases in each season (one-way analysis of variance, ANOVA, $P < 0.05$; Bonferroni, $P < 0.05$). Inter-seasonal variations of a peptidase activity in the same fraction of semen collected from the ductus deferens are indicated by different *letters* (one-way analysis of variance, ANOVA, $P < 0.05$; Bonferroni, $P < 0.05$). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ indicate differences of a peptidase activity in the same fraction between the semen collected from the ductus deferens throughout the year and that collected from the uterus in the winter (unpaired, two-side Student's *t* test). Number of animals is in *parentheses*

APN-PS, CAP, DPPIV and PAP-I in SF and MF in semen from the ductus deferens and from the uterus. The levels of examined peptidase activities in semen collected from the ductus deferens in all seasons and semen collected from the uterus in austral winter revealed that APN-PI was the most pronounced activity in SF and MF; in fact, APN-PI (SF and MF), APN-PS (SF) and APB (SF) activities were marked in semen samples collected from male and female, except APN-PI (SF) in semen from the uterus and APN-PS (SF) in semen from ductus deferens collected in summer. APA, APB, APN-PI, APN-PS and POP activities in SF as well as APN-PI, APN-PS and DPPIV in MF varied seasonally. In semen collected from the ductus deferens, APB and POP activities in SF were higher in austral summer, while APA activity in SF and MF was higher in austral winter than in other seasons. In SF and MF of semen collected from the ductus deferens, APN-PS activity was higher in austral winter than in other seasons, while APN-PI was higher in austral winter than in spring. DPPIV activity in MF was higher in austral autumn than in summer. Comparisons between semen from both origins revealed differences in APA (SF and MF), APN-PI (SF), APN-PS (SF and MF), CAP (MF), DPPIV (SF and MF), PAP-I (SF and MF), PIP (SF) and POP (SF). During austral spring, APA (SF and MF), APN-PS (SF), DPPIV (MF), PAP-I (SF and MF), PIP (SF) and POP (SF) activities were lower, while APN-PI (SF) was higher in the semen from the ductus deferens than in the semen from the uterus. During austral summer, APN-PI (SF) was higher and APA (SF and MF), APN-PS (SF) and DPPIV (SF and MF) were lower in semen collected from the ductus deferens than in semen from the uterus. During austral autumn, APA (SF and MF), APN-PS (SF), CAP (MF), DPPIV (MF), PAP-I (SF and MF), and PIP (SF) were lower, while APN-PI (SF) was higher in semen collected from the ductus deferens than in semen from the uterus.

Discussion

General considerations

The semen collected from the ductus deferens did not have secretions from renal sexual segment (because the ureter is not continuous with the ductus deferens) (Sever et al. 2002), whereas those collected from the females did. However, semen from the ductus deferens would have mixed with fluids 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; Sever 2004; Akbarsha et al. 2005). Therefore, the differences between the peptidase activities in the ductus deferens compared to that of the uterus could be originated by the influence of different secretions in these locations. Furthermore, the site and duration of semen storage in the uterus could also be responsible for these differences and thus the differences of peptidase activities between semen collected from male and female could be artifacts of seminal degradation in the female reproductive tract over time or factors that help the viable spermatozoa storage in the posterior uterus of *C. d. terrificus*. Additional investigations are needed to evaluate all these hypotheses and concerning the females the present study only demonstrated that the activities of the peptidases differ from males. Overall, the profiles of seasonal variations of the activities of the peptidases observed in the present study in semen collected from male evidenced their relationships with male reproductive cycle and with the seminal quality of *C. d. terrificus*.

In general, the differences of APN-PI and also APB and APN-PS activities, compared to the other peptidase activities in most part of the seasons, unveil their relevance to the seminal physiology of *C. d. terrificus*. Interestingly, these enzymes may have no role in the in vitro process of liquefaction of the semen coagulum of *C. d. terrificus*. In spite of the high levels of aminopeptidase activities, we observed that the rattlesnake semen has no viscosity changes, even with twice the time required for the liquefaction of human semen (60 min at room temperature) (WHO 1999). The absence of a prostate gland, seminal vesicles and bulbourethral and urethral glands in squamates (Sever 2004) may be determinant in the liquefying inability. The liquefaction of human semen is known to occur mainly by the prostate-specific antigen, a serine protease in the seminal plasma, which promotes the proteolytic fragmentation of the seminal plasma coagulum (Koistinen et al. 2002), this event is also aided by aminopeptidase activities (Chatterjee et al. 1997). Occasionally, human semen liquefaction does not occur and mechanical mixing or enzyme treatment (e.g. bromelain, 1 mg/mL) can be used for this purpose (WHO 1999). Independently of the season in which samples were obtained, mechanical mixing of *C. d. terrificus* semen (collected from the ductus deferens or from the uterus) was not sufficient to promote liquefaction. Moreover, bromelain (1 mg/mL) and hyaluronidase (0.04 mg/mL) did not alter semen viscosity. These data indicate that the semen probably liquefies only within the reproductive tract.

Seminal peptidases in the ductus deferens

Regarding the semen collected from male, the two most prominent peptidase activities, APN-PI (SF and MF) and APN-PS (SF and MF), together with APA (SF and MF), had a common pattern of increase during the dry season (austral autumn/winter), reaching a significant level in winter compared to other seasons. Furthermore, APA (SF and MF) and APN-PS (SF and MF) were lower in summer than in winter. These patterns agree with the occurrence of integrative changes among these aminopeptidase activities, hormonal levels, male reproductive cycle and spermatozoa viability (reduction of motility of spermatozoa during the winter). In *C. d. terrificus*, during the austral winter, levels of testosterone decrease and an increase in spermatozoa defects occur compared to austral summer (Zacariotti 2004). Lower APN activity in the semen suggests a better spermatozoon quality, since an increase of APN activity is correlated to sperm inviability in humans (Irazusta et al. 2004). Considering that APA cleaves Ang II, the effects of the hydrolysis of Ang II by APA could result in a higher sperm capacitation in the periods of pre-mating and mating, and a lower sperm capacitation in post-mating. In addition, in the semen collected from the ductus deferens, APB and POP activities were higher in austral summer, peak of spermatogenesis (Salomão and Almeida-Santos 2002), than in other seasons, suggesting the relation of these enzymes with spermatogenesis. APB and POP hydrolyze BK, a peptide that stimulates the motility of human sperm (Schill et al. 1989) and thus high levels of these peptidases increase the inactivation of BK and contribute to the reduction of spermatozoa motility of *C. d. terrificus* during the peak of spermatogenesis. Finally, the increase of POP activity in human semen has been related to necrozoospermia (Valdivia et al. 2004) and thus the increase of POP activity in the semen collected from the ductus deferens in austral summer also suggests a higher necrozoospermia immediately before the mating period of *C. d. terrificus*. Also, the increased POP activity in semen collected from the ductus deferens in summer implies a decrease of TRH and FPP levels with consequent decrease of capacitation and fertilizing ability of uncapacitated spermatozoa and maintenance of capacitation and acrosome reaction in capable spermatozoa (Fraser et al. 2001). However, the number of spermatozoa of *C. d. terrificus* is increased in austral summer and autumn compared to austral winter (Almeida-Santos et al. 2004a). Taken together these data agree that the peak

of spermatozoa storage in the ductus deferens is combined with the period that females are attractive (Almeida-Santos and Orsi 2002). Overall, these data provide additional evidences that spermatozoa gain fertilizing ability after spermiogenesis in summer, which contribute to justify a temporal dissociation between the peak of spermatogenesis and mating in *C. d. terrificus*.

Comparison between seminal peptidases in the posterior uterus and in the ductus deferens

Regarding the comparisons of peptidase activity levels between semen from the ductus deferens and semen from the uterus, APA (SF and MF), APB (SF), APN-PI (MF), CAP (SF and MF), DPPIV (SF and MF), PAP-I (SF and MF), PIP (SF) and POP (SF) had similar or higher levels in the semen collected from the uterus than in the semen collected from the ductus deferens in winter. This profile could be related to two opposing possibilities: (1) artifacts of male seminal degradation in the female reproductive tract over time or (2) the maintenance of quiescent spermatozoa. In the latter case, the reduced motility and capacitation during storage in the uterus could be reverted during the fertilization period. Although the present study was unable to clarify this controversy, some points evidenced by the comparisons of peptidase activity levels between semen from the ductus deferens and semen from the uterus should be mentioned, e.g. the remarkable opposite differences of APN-PI and APN-PS activities in SF between the semen collected from the ductus deferens in austral summer (peak of spermatogenesis) and semen collected from the uterus, indicating a crucial role of these enzymes in seminal function and physiological adaptation to the maintenance of spermatozoa during the storage in the uterus. Otherwise, activity levels of APN-PI (SF) in all seasons and APN-PS (SF and MF) in winter were higher in semen from the ductus deferens than in semen from the uterus, which agree with the hypothesis that factors provided by the female can increase spermatozoon viability (Almeida-Santos and Salomão 1997), since lower APN activity in semen from females suggests a better spermatozoon quality (Irazusta et al. 2004). In this sense, the maintenance of spermatozoa fertility in the uterus, during the storage, would be in part a result of the decrease of APN-PI (SF) and the increase of APA (SF and MF); it was noteworthy that APA activity in semen from the ductus deferens increased gradually until its peak in winter, when semen stored in the female had a similar level. Furthermore, the observation that semen collected from the ductus deferens had decreased activity levels of POP in spring relative to semen from the uterus also contribute to justify a temporal dissociation between ovulation and mating, since the decrease of POP activity (increase of TRH and FPP) leading to the inhibition of spontaneous loss of acrosome (Fraser et al. 2001) coincides with ovulation. Consequently, if mating occurred coincident with ovulation in this species, the spermatozoa would be less able than those stored in the uterus. In this sense, the storage in the uterine environment could be a necessary physiological factor for the fertilization process. However, lower activity levels of PAP-I in the semen from the ductus deferens throughout the year relative to the semen from the uterus were not compatible with this hypothesis, considering that TRH and FPP contents could also be potentially increased by the reduction of PAP-I throughout the year. Until now, spermatozoa storage has been considered an adaptation of snakes from temperate regions (Shine 1977), leading to the assumption that *C. d. terrificus* originated from a temperate region and maintained a similar pattern of storage, even in a location (tropical region) where this process is unnecessary (Almeida-Santos et al. 2004b).

In conclusion, the present work highlights the seasonal variation among peptidase activity levels in semen collected from the ductus deferens, which differs from that stored in the posterior portion of the uterus, reflecting a modulatory role of these peptidases in the functionality of *C. d. terrificus* semen.

Acknowledgments The authors thank Valdir José Germano for his efficient technical assistance. This investigation was supported by a Research Grant 04/14971-9 from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil). C.E.M. (05/03745-0) and S.C.Y. (07/06829-6) were sponsored by FAPESP.

References

Akbarsha MA, Tamilarasan V, Kadalmani B, Daisy P (2005) Ultrastructural evidence for secretion from the epithelium of ampulla ductus deferentis of the fan-throated lizard *Sitana ponticeriana* Cuvier. J Morphol 266:94–111

Almeida-Santos SM, Orsi AM (2002) Reproductive cycle in *Crotalus durissus* and *Bothrops jararaca* (Serpentes, Viperidae): morphology and oviductal function. Rev Bras Reprod Anim 26:109–112

Almeida-Santos SM, Salomão MG (1997) Long-term sperm storage in the neotropical rattlesnake *Crotalus durissus terrificus* (Viperidae: Crotalinae). Jpn J Herpetol 17:46–52

Almeida-Santos SM, Laporta-Ferreira IL, Antoniazzi MM, Jared C (2004a) Sperm storage in males of the snake *Crotalus durissus terrificus* (Crotalinae: Viperidae) in southeastern Brazil. Comp Biochem Physiol A Mol Integr Physiol 139:169–174

Almeida-Santos SM, Abdalla FMF, Silveira PF, Yamanouye N, Breno MC, Salomão MG (2004b) Reproductive cycle of the neotropical *Crotalus durissus terrificus*. I. Seasonal levels and interplay between steroid hormones and vasotocinase. Gen Comp Endocrinol 139:143–150

Amory JK, Bremner WJ (2003) Regulation of testicular function in men: implications for male hormonal contraceptive development. J Steroid Biochem Mol Biol 85:357–361

Assinder SJ, Rezvani A, Nicholson HD (2002) Oxytocin promotes spermiation and sperm transfer in the mouse. Int J Androl 25:19–27

Barret AJ, Rawlings ND, Woessner JF (1998) Handbook of proteolytic enzymes. Academic Press, London

Bergmeyer HU, Brent E (1972) Assay with pyruvate and NADH. In: Bergmeyer HU (ed) Methods in enzymatic analysis, vol 2. Academic Press, London, pp 574–577

Bishop JE (1959) A histological and histochemical study of the kidney tubule of the common garter snake, *Thamnophis sirtalis*, with special reference to the sexual segment in the male. J Morphol 104:307–357

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254

Chatterjee S, Laloraya M, Kumar PG (1997) Free radical-induced liquefaction of ejaculated human semen: a new dimension in semen biochemistry. Arch Androl 38:107–111

Davison JM, Sheills EA, Philips PR, Barron WM, Lindheimer MD (1993) Metabolic clearance of vasopressin and an analogue resistant to vasopressinase in human pregnancy. Am J Physiol 264:F348–F353

El-Haggar S, El-Ashmawy S, Attia A, Mostafa T, Roaiah MM, Fayez A, Ghazi S, Zohdy W, Roshdy N (2006) Beta-endorphin in serum and seminal plasma in infertile men. Asian J Androl 8:709–712

Fernández D, Valdivia A, Irazusta J, Ochoa C, Casi L (2002) Peptidase activities in human semen. Peptides 23:461–468

Fraser LR, Pondel MD, Vinson GP (2001) Calcitonin, angiotensin II and FPP significantly modulate mouse sperm function. Mol Hum Reprod 7:245–253

Gasparello-Clemente E, Casis L, Varona A, Gil J, Irazusta J, Silveira PF (2003) Aminopeptidases in visceral organs during alterations in body fluid volume and osmolality. Peptides 24:1367–1372

Green CM, Cockle SM, Watson PF, Fraser LR (1996) Fertilization promoting peptide, a tripeptide similar to thyrotrophin-releasing hormone, stimulates the capacitation and fertilizing ability of human spermatozoa in vitro. Hum Reprod 11:830–836

Himmelhoch SR (1970) Leucine aminopeptidase from swine kidney. Methods Enzymol 19:508–513

Irazusta J, Valdivia A, Fernández D, Agirregoitia E, Ochoa C, Casis L (2004) Enkephalin-degrading enzymes in normal and subfertile human semen. J Androl 25:733–739

Koistinen H, Soini T, Leinonen J, Hyden-Granskog C, Salo J, Halttunen M, Stenman UH, Seppälä M, Koistinen R (2002) Monoclonal antibodies, immunofluorometric assay, and detection of human semenogelin in male reproductive tract: no association with in vitro fertilizing capacity of sperm. Biol Reprod 66:624–628

Kugler P (1982) Aminopeptidase A is angiotensinase A. II. Biochemical studies on aminopeptidase A and M in rat kidney homogenate. Histochemistry 74:247–261

Langlada FG, Santos S, Ferreira ILL (1994) Tecniques of artificial insemination in *Crotalus durissus terrificus* (Viperidae: Crotalinae). Braz J Vet Res Anim Sci 31:141–144

Mededovic S, Fraser LR (2005) Mechanisms of action of angiotensin II on mammalian sperm function. Reproduction 129:211–218

Mizutani S, Goto K, Nomura S, Ino K, Goto S, Kikkawa F, Kurauchi O, Goldstein G, Tomoda Y (1993) Possible action of human placental aminopeptidase N in feto-placental unit. Res Commun Chem Pathol Pharmacol 82:65–80

Nilson G, Andrén C (1982) Function of renal sex secretion and male hierarchy in the adder *Vipera berus*, during reproduction. Horm Behav 16:404–413

O'Cuinn G, O'Connor B, Elmore M (1990) Degradation of thyrotropin-releasing hormone and luteinising hormone-releasing hormone by enzymes of brain tissue. J Neurochem 54:1–13

O'Hara BF, Donovan DM, Lindberg I, Brannock MT, Ricker DD, Moffatt CA, Klaunberg BA, Schindler C, Chang TS, Nelson RJ, Uhl GR (1994) Proenkephalin transgenic mice: a short promoter confers high testis expression and reduced fertility. Mol Reprod Dev 38:275–284

Salomão MG, Almeida-Santos SM (2002) The reproductive cycle in male neotropical rattlesnake (*Crotalus durissus terrificus*). In: Schuett GW, Höggren M, Douglas ME, Greene HW (eds) Biology the vipers. Eagle Mountain, Utah

Sastry BV, Janson VE, Owens LK (1991) Significance of substance P- and enkephalin-peptide systems in the male genital tract. Ann NY Acad Sci 632:339–353

Schill WB, Miska W, Parsch EM, Fink E (1989) Significance of the kallikrein–kinin system in andrology. In: Fritz H, Schmidt I, Dietze GD (eds) The kallikrein–kinin system health and disease. Limbach Verlag, Braunschweig, pp 171–203

Sever DM (2004) Ultrastructure of the reproductive system of the black swamp snake (*Seminatrix pygaea*). IV. Occurrence of an ampulla ductus deferentis. J Morphol 262:714–730

Sever DM, Stevens RA, Ryan TJ, Hamlett WC (2002) Ultrastructure of the reproductive system of the black swamp snake (*Seminatrix pygaea*). III. Sexual segment of the male kidney. J Morphol 252:238–254

Shine R (1977) Reproduction in Australian elapid snakes. II. Female reproductive cycles. Aust J Zool 25:655–666

Siegel DS, Sever DM (2008) Sperm aggregations in female *Agkistrodon piscivorus* (Reptilia: Squamata): a histological and ultrastructural investigation. J Morphol 269:189–206

Siviter RJ, Cockle SM (1995) Peptides related to thyrotrophin-releasing hormone are degraded in seminal plasma by an enzyme similar to prolyl endopeptidase. J Endocrinol 144:61–66

Subirán N, Agirregoitia E, Valdivia A, Ochoa C, Casis L, Irazusta J (2008) Expression of enkephalin-degrading enzymes in human semen and implications for sperm motility. Fertil Steril 89:1571–1577

Valdivia A, Irazusta J, Fernández D, Múgica J, Ochoa C, Casis L (2004) Pyroglutamyl peptidase I and prolyl endopeptidase in human semen: increased activity in necrozoospermia. Regul Pept 122:79–84

Vinson GP, Mehta J, Evans S, Matthews S, Puddefoot JR, Saridogan E, Holt WV, Djahanbakhch O (1996) Angiotensin II stimulates sperm motility. Regul Pept 67:131–135

Volsoe H (1944) Structure and seasonal variation of the male reproductive organs of *Vipera berus* (L.). Spolia Zoologica Musei Hauniensis V. Skrifter 5:1–157

World Health Organization (1999) WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction. The Press Syndicate of the University of Cambridge, Cambridge

Yamanouye N, Silveira PF, Abdalla FMF, Almeida-Santos SM, Breno MC, Salomão MG (2004) Reproductive cycle of the neotropical *Crotalus durissus terrificus*. II. Establishment and maintenance of the uterine muscular twisting, a strategy for long-term sperm storage. Gen Comp Endocrinol 139:151–157

Zacariotti RL (2004) Estudo longitudinal do espermograma e dos níveis de testosterona sérica de cascavel (*Crotalus durissus terrificus*, Laurenti, 1768) proveniente da natureza do Estado de São Paulo. 80 f. Dissertação (Mestrado em Reprodução Animal), Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo