

## Purification and partial characterization of proteinase inhibitors of equine seminal plasma

*André Belico Vasconcelos<sup>1,2</sup>, Alexandre Martins Costa Santos<sup>3</sup>,  
Jamil Silvano Oliveira<sup>4</sup>, Monique de Albuquerque Lagares<sup>5</sup>  
Marcelo Matos Santoro<sup>4</sup>*

*<sup>2</sup>Institute of Veterinary "José Caetano Borges",  
University of Uberaba, <sup>3</sup>Department of Physiology Science, Federal  
University of Espirito Santo, <sup>4</sup>Department of Biochemistry Immunology,  
<sup>5</sup>Department of Veterinary Clinic and Surgery, Federal University  
of Minas Gerais, Brazil*

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### SUMMARY

The aims of the study were 1/ to isolate and identify equine seminal plasma proteinase inhibitors, 2/ to evaluate their inhibitory potential, and 3/ to test a correlation between protein concentration in seminal plasma supernatant (obtained after precipitation with 36% ammonium sulfate) and stallion sexual maturity. Seminal plasma proteins obtained from six stallions were chromatographed in a Superose 12 (FPLC system) column followed by C<sub>18</sub> HPLC reverse-phase. Inhibition of trypsin amidase activity was evaluated in the collected fractions. Active proteins with a molecular mass of 6.3-7.0 kDa were identified using mass spectrometry. The older stallions showed a reduction in total seminal plasma protein concentration, but had similar concentrations of proteinase inhibitors (0.28±0.10 mg/ml) in seminal plasma

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<sup>1</sup>Corresponding author: Institute of Veterinary „José Caetano Borges”, Uberaba, Minas Gerais, Av. Tutunas 720, Postal code 38061-500, Brazil, e-mail: andre\_belico@yahoo.com.br

supernatant. Different proteinase inhibitor isoforms were found in semen of all stallions which suggests that the isoforms may be used as biomarkers of individual animals. *Reproductive Biology*, 2009 **9** 2: 151–160.

**Key words:** serine-proteinase inhibitor, seminal plasma, stallion, sexual maturity, equine

## INTRODUCTION

Spermatozoa acquire the ability to fertilize an egg during a complex, sequentially-ordered process known as post-testicular sperm maturation [21]. Some seminal proteins are serine-proteinase inhibitors which are important natural tools for regulating the proteolytic activity of their target proteinase or for signaling receptor interactions [2, 16]. Proteinase inhibitors have been discovered and characterized [2] in seminal plasma of bulls [14], mice [13] and guinea pigs [20].

The study of seminal plasma proteinase inhibitors may help to understand the changes that occur in protein functions and protein-protein interactions. The latter phenomena can modulate sperm functions such as hyperactivation and acrosome reaction [1, 16]. Proteinase inhibitors – acting as decapacitating factors [3] – may prevent *in vitro* sperm penetration of zona pellucida in mammals [6, 9]. Since these inhibitors bind to the sperm acrosomal region, it is suggested that they may play a role in the fertilization process [13]. The aims of the study were 1/ to isolate and identify equine seminal plasma proteinase inhibitors, 2/ to evaluate their inhibitory potential, and 3/ to test a correlation between protein concentration in seminal plasma supernatant (obtained after precipitation with 36% ammonium sulfate) and stallion sexual maturity.

## MATERIALS AND METHODS

*Animals.* Ejaculates of six stallions (5-20 years old) were collected using an artificial vagina (model “Hannover”). Semen samples were evaluated for progressive motility with a bright field microscopy (100×). Ejaculates

containing a minimum of 50% of spermatozoa with progressive motility were used in the study. All samples were cooled to +5°C with a cooling rate of 1°C/min; such a rate does not induce a cold-shock effect [11, 15].

*Protein purification.* In order to obtain seminal plasma proteins, semen was centrifuged for 30 min at +4°C (600×g). Then, the supernatant was brought to 36% (wt/vol) saturation with solid ammonium sulphate adjusted to pH 2.0 with 6N HCl, stirred for 30 min, and allowed to stand at 0°C for 30 min [14]. The samples were centrifuged again (600×g, 4°C, 30 min), the supernatant was dialyzed for 48 h against several volumes of 0.5% acetic acid using exclusion membrane 1000 Da, and then lyophilized. The lyophilized protein samples were dissolved in 500 µl of 25 mM Tris-HCl (Sigma, USA) buffer, pH 7.4. Protein concentration was measured with Coomassie Brilliant Blue BG-250 [4].

Trypsin inhibitors were further purified by gel filtration chromatography using a Superose 12 HR 10/30 column equilibrated with 25 mM Tris-HCl, pH 7.4 in a fast performance liquid chromatography (FPLC-system). The fractions containing trypsin inhibitory activity were then applied to a reverse-phase C<sub>18</sub> column Shim-pack CLC-ODS (M) and subjected to a reversed phase high performance liquid chromatography (RP-HPLC). Following the RP-HPLC, the inhibitors were identified in a mass spectrometer (Q-ToF Micromass) containing an electrospray interface (ESI-MS) and data handling system (Masslink 3.5). Aliquots (20 µl) of trypsin inhibitor fractions were added to a solvent mixture of water/acetonitrile 50/50 (V/V) plus 0.1% formic acid; up to 25-50 µmol of protein was analyzed. Samples were injected directly into the electrospray source *via* a loop injector at a solvent flow rate of 10 ml/min. The data were collected using the capillary voltage of 2000 V and sample cone voltage of 15.0 V.

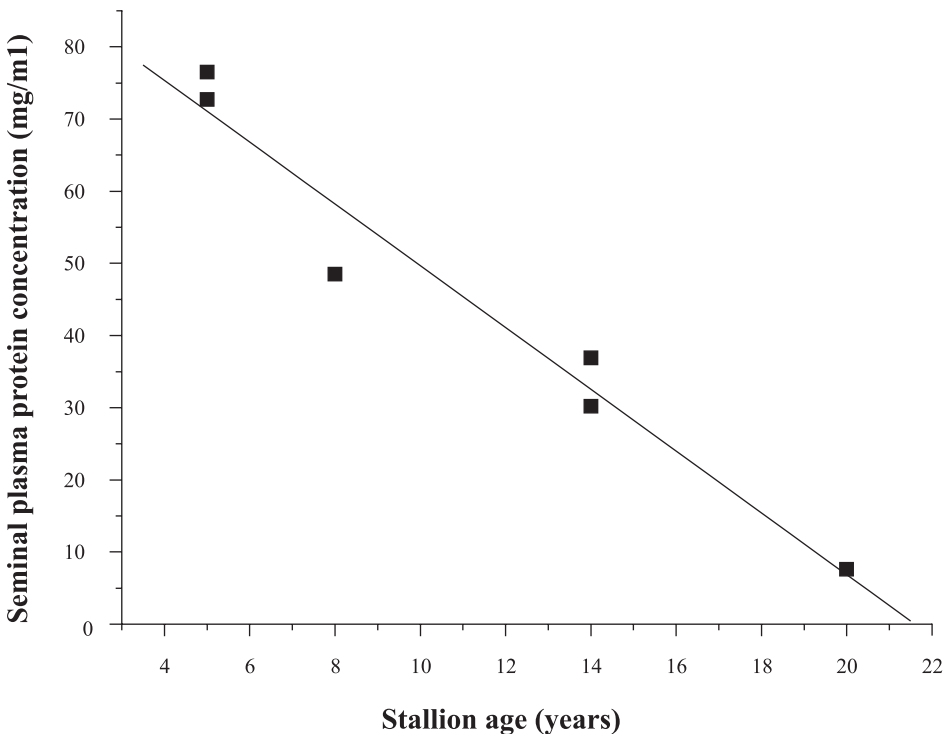
*Proteinase inhibitory activity.* Trypsin activity was used to evaluate proteinase inhibitory activity. The trypsin activity was assessed spectrophotometrically (UV-visible recording spectrophotometer Shimadzu-160A) at 410 nm using a synthetic substrate N-alpha-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) in 25 mM (Tris-HCl), pH 7.4 at 37°C. Each 1 ml assay contained 3 µg/ml trypsin, 55 µM DL-BAPNA, and various amounts of buffered supernatant protein fractions [3, 21].

*Statistical analysis.* The linear regression between seminal plasma protein concentration and stallion age (fig. 1) was calculated.

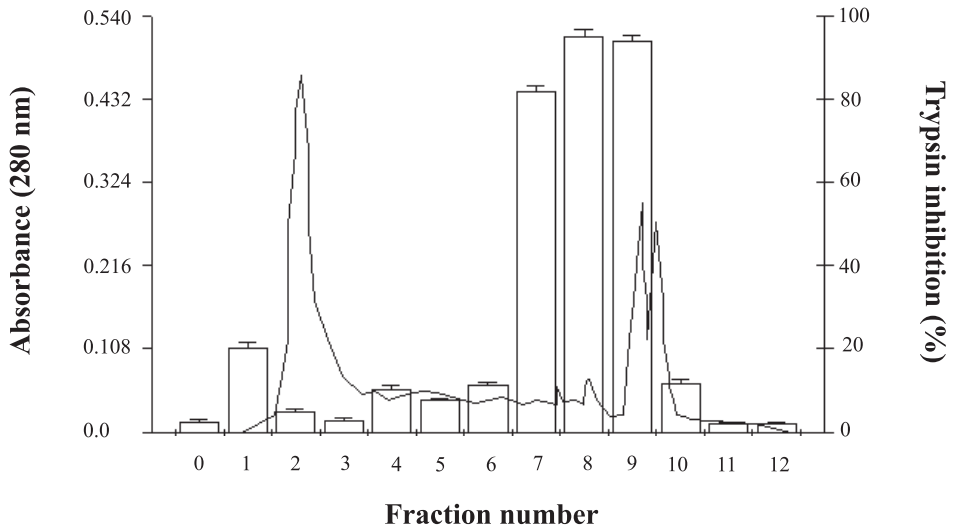
## RESULTS

*Regression between protein concentration and stallion age.* After treatment with 36% of ammonium sulfate, the Bradford [4] assay was used to determine the seminal plasma supernatant protein concentration. A negative regression was found between stallion age and supernatant protein concentrations (fig. 1).

*Gel filtration of proteinase inhibitors.* After determination the seminal plasma supernatant protein, seminal plasma samples were submitted to gel



*Figure 1.* Regression between protein concentration in seminal plasma supernatant after ammonium sulfate precipitation and stallion age; regression equation:  $Y=A+Bx$ ; parameters (value, error):  $A=92.52, 5.64$ ;  $B=-4.283, 0.459$ ;  $R^2=0.978$ ;  $p<0.001$



*Figure 2.* Fast performance liquid chromatography (FPLC) of stallion seminal proteins using a Superose 12 gel filtration column. Absorbance at 280 nm is presented as a line and the peaks represent different proteins; trypsin of inhibitory activity (mean $\pm$ SD) is presented as bars.

filtration (Superose 12) chromatography in FPLC-system. Twelve fractions were collected and the proteinase inhibitor activity was found in fractions 7, 8 and 9 with an inhibition of app. 97% (fig. 2). This was observed in all studied animals. Protein concentrations in the combined fractions 8 and 9

*Table 1.* Protein concentration after gel filtration in combined fractions No. 8 and 9

Animal	Age (years)	Protein concentration (mg/ml)
1	14	0.27
2	5	0.25
3	20	0.45
4	5	0.13
5	14	0.30
6	8	0.26

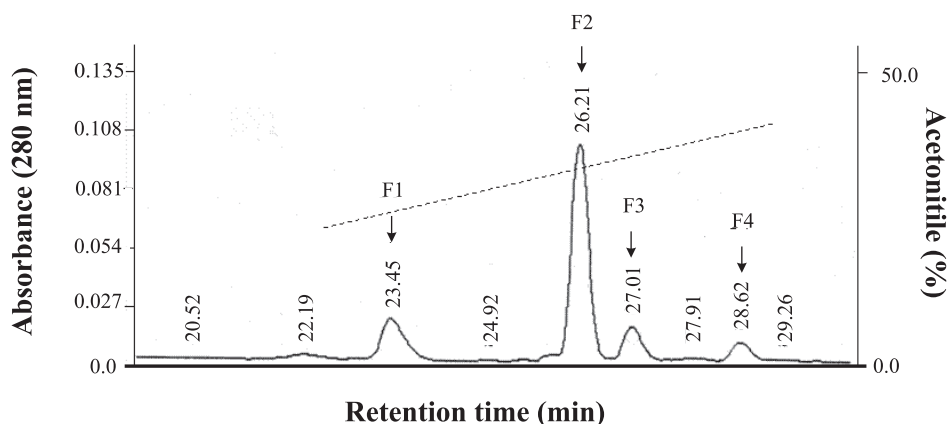


Figure 3. Reverse-phase high performance liquid chromatography (RP-HPLC) of combined fractions 8 and 9 obtained after gel filtration (see: fig. 2). Elution was carried out with a linear gradient of 0-80% acetonitrile/0.1%TFA for 50 minutes. The dotted line represents acetonitrile gradient.

showed individual variability (tab. 1), however, the trypsin inhibitor concentration was similar in each stallion ( $0.28 \pm 0.10$  mg/ml).

*HPLC chromatography of proteinase inhibitors.* The fractions containing trypsin inhibitors (fractions 8 and 9) were combined and applied to a reverse phase  $C_{18}$  column in order to obtain a sample of high purity. Four protein fractions were obtained (fig. 3), and the percentage of peak area and inhibitory activity are presented in Table 2.

Table 2. Characterization of protein fractions obtained by RP-HPL chromatography

Retention time (min)	Fraction number	Peak area (%)	Trypsin inhibition (%)	Inhibition/peak area
23.45	F1	17.5	97	5.54
26.21	F2	64.2	50	0.78
27.01	F3	13.9	34	2.45
28.62	F4	4.4	20	4.54

Table. 3. Molecular mass (Da; mean±SD) of trypsin inhibitors of the F1 fraction detected by mass spectrometry (ESI-MS) in individual stallions

Stallion	Ion peaks detected by ESI-MS	
1	6925.14+0.02	-----
2	6772.65+0.14	7227.48+0.38
3	6732.42+0.01	7030.19+0.50
4	6372.75+0.03	7226.93+0.55
5	6372.71+0.05	7029.42+0.39
6	6372.26+0.55	-----

F1 fraction was obtained after RP-HPLC; only one sample from each of the six animals was used for mass spectrometry evaluation

*Mass spectrometry results.* The ESI-MS spectra showed two distinct distribution patterns of ion peaks. The presence of one or two trypsin inhibitors of the F1 fraction seminal plasma is shown in Table 3; each stallion contained inhibitors of different molecular masses in seminal plasma.

## DISCUSSION

In the present study, a negative regression between stallion age and semen supernatant protein concentration was found. According to Strzezek et al. [17], there are qualitative changes in protein profiles of boar seminal plasma in relation to age and quantitative changes in response to variation of physiological processes. In 4.5 year-old stallions, the biochemical components of the seminal plasma such as glycerylphosphorylcholine, ergothioneine and total protein are significantly lower than those of mature stallions [12, 18]. Therefore, the variation in protein concentration observed in the present study might be related to changes in seminal plasma components during equine sexual maturation.

The proteinase inhibitor was identified in seminal plasma of boars [10], bulls [14] and stallions [5]. A protein complex displaying proteinase inhibitory properties (800 kDa) and composed of different polypeptides (11 to 30 kDa)

was observed by Fillenberge et al. [5] in horse seminal plasma. The same authors reported the presence of a proteinase (6.5 kDa) trypsin inhibitor. The present study, using mass spectrometry, showed the presence of numerous proteinase inhibitors characterized by a variation in molecular mass (6.3-7.0 kDa). The numerous peaks observed after chromatography suggest the presence of proteinase inhibitor isoforms. The protein variability may be related to the sexual maturation process [17] since proteomic changes may generate different isoforms during this process [8]. Thus, this suggests the possibility to use proteinase inhibitors as biomarkers of stallion semen quality [7].

Some serine proteinase inhibitors bind to the acrosomal region of the sperm, and their release during *in vitro* or *in utero* incubation suggests that they may play a role in the fertilization process [13, 19]. Further studies are necessary to evaluate the role of the inhibitors in the fertilization process as well as to elucidate mechanisms related to their production and structural changes.

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## REFERENCES

1. Bedford JM **1970** Sperm capacitation and fertilization in mammals. *Biology of Reproduction* **2** 128-158.
2. Bode W, Huber R **1992** Natural protein proteinase inhibitors and their interaction with proteinases. *European Journal of Biochemistry* **204** 433-451.
3. Boettger-Tong H, Aarons B, Biegler T, Poirier GR **1992** Competition between zonae pellucidae and a proteinase inhibitor for sperm binding. *Biology of Reproduction* **47** 716-722.
4. Bradford M **1976** Rapid and sensitive method for the quantization of microgram quantities of proteins utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248-254.

5. Fillenberge RV, Zweifel HR, Gruenig G, Pellegrini A **1985** Proteinase inhibitors of horse seminal plasma. *Biological Chemistry Hoppe-Seyler* **366** 705-712.
6. Fraser LR **1982** p-Aminobenzamidine, an acrosin inhibitor, inhibits mouse sperm penetration of the zona pellucida but not the acrosome reaction. *Journal of Reproduction and Fertility* **65** 185-194.
7. Fraser L, Wysocki P, Ciereszko A, Plucienniczak G, Kotłowska M, Kordan W, Wojtczak M, Dietrich G, Strzeżek J **2006** Application of biochemical markers for identification of biological properties of animal semen. *Reproductive Biology* **6** 5-20.
8. Hachey LD, Chaurand P **2004** Proteomics in reproductive medicine: the technology for separation and identification of proteins. *Journal of Reproductive Immunology* **63** 61-73.
9. Jansen S, Jones R, Jenneckens I, Marschall B, Kreigesmann B, Coadwell J, Brenig B **1998** Site-directed mutagenesis of boar proacrosin reveals residues involved in binding of zona pellucida glycoproteins. *Molecular Reproduction and Development* **51** 184-192.
10. Jelínková P, Manásková P, Tichá M, Jonáková V **2003** Proteinase inhibitors in aggregated forms of boar seminal plasma proteins. *International Journal of Biological Macromolecules* **32** 99-107.
11. Kayser JP, Amann RP, Shideler RK, Squires EL, Jasko DJ, Pickett BW **1992** Effects of linear cooling rate on motion characteristics of stallion spermatozoa. *Theriogenology* **38** 601-614.
12. Kosiniak K, Bittmar A **1987** Analysis of the physiological processes connected with sexual maturation of stallions. (in Polish) *Polskie Archiwum Weterynaryjne* **27** 5-21.
13. Lai ML, Chen SW, Chen YH **1991** Purification and characterization of trypsin inhibitors from mouse seminal vesicle secretion. *Archives of Biochemistry and Biophysics* **290** 265-271.
14. Lewis RV, Agustin JS, Kruggel W, Lardy HA **1985** The structure of caltrin, the calcium-transport inhibitor of bovine seminal plasma. *Proceedings of the National Academy of Sciences of USA* **82** 6490-6491.
15. Moore AI, Squires EL, Bruemmer JE, Graham JK **2006** Effect of cooling rate and cryoprotectant on the cryosurvival of equine spermatozoa. *Journal of Equine Veterinary Science* **26** 215-218.
16. Perreault SD, Zirkin BR, Rogers BJ **1982** Effect of trypsin inhibitors on acrosome reaction of guinea pig spermatozoa. *Biology of Reproduction* **26** 343-351.
17. Strzeżek J, Wysocki P, Kordan W, Kuklinska M, Mogielnicka M, Soliwoda D, Fraser L **2005** Proteomics of boar seminal plasma-current studies and possibility of their application in biotechnology of animal reproduction. *Reproductive Biology* **5** 279-290.
18. Töpfer-Petersen E, Ekhlasi-Hundrieser M, Kirchhoff C, Leeb T, Sieme H **2005** The role of stallion seminal proteins in fertilization. *Animal Reproduction Science* **89** 159-170.
19. Tschesche HH, Wittig B, Decker G, Muller-Esterl W, Fritz H **1982** A new acrosin inhibitor from boar spermatozoa. *European Journal of Biochemistry* **126** 99-104.

20. Winnica ED, Novella ML, Dematteis A, Coronel EC **2000** Trypsin/acrosin inhibitor activity of rat and guinea pig caltrin proteins structural and functional studies. *Biology of Reproduction* **63** 42-48.
21. Yanagimachi R **1988** Mammalian fertilization. *The Physiology of Reproduction*, pp135-185. Eds Knobil,E and Neil, JD, Raven Press, New York.