Published-Ahead-of-Print December 8, 2005, DOI:10.2164/jandrol.05170
Journal of Andrology, Vol. 27, No. 3, May/June 2006

DOI: 10.2164/jandrol.05170

Dissecting the Protective Effect of the Seminal Plasma Spermadhesin PSP-I/PSP-II on Boar Sperm Functionality

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Received for publication September 8, 2005; accepted for publication November 28, 2005.

Abstract

To dissect the protective activity of PSP-I/PSP-II, the effect of the isolated subunits PSP-I and PSP-II and their affinity-purified tryptic peptide and glycan fractions on the viability, mitochondrial activity, and motility of highly diluted boar spermatozoa was investigated. High dilution exerted a negative effect on control spermatozoa. Incubation of spermatozoa with PSP-I/PSP-II or with its PSP-II subunit had a protective effect on sperm functionality, high mitochondrial membrane potential, and sperm motility. These effects were less pronounced when spermatozoa were incubated with the PSP-I subunit. It was noteworthy that motility was abolished by incubation of spermatozoa with isolated PSP-I. Trypsin-degraded PSP-I/PSP-II, PSP-I, and PSP-II reproduced the effects of the native proteins. Incubating spermatozoa with the glycan-depleted tryptic-peptide fraction of PSP-I/PSP-II for 5 hours preserved a higher percentage of viable spermatozoa than when sperm was incubated for the same time with the native heterodimer, trypsin-digested PSP-I/PSP-II, the glycan fraction or without added proteins. However, sperm motility decreased as the concentration of added peptide fraction increased. On the other hand, spermatozoa incubated with the glycan fraction showed lower values than spermatozoa incubated with the peptide fraction. We concluded that the subunits of the PSP-I/PSP-II heterodimeric spermadhesin exert different activities on sperm functions. The

finding that the beneficial effect of the native PSP-I/PSP-II on the functionality of highly diluted boar spermatozoa is largely preserved in its isolated PSP-II subunit and does not appear to require the glycan moiety points to a peptide moiety as a potential sperm function-preserving additive of highly diluted boar spermatozoa.

Key words: Boar seminal plasma, spermadhesin PSP-I/PSP-II, protective effect on sperm function, sperm survival, highly diluted spermatozoa

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The seminal plasma, consisting of secretions from the testes, epididymis, and the accessory sex glands, contains a variety of factors (amino acids, lipids, fatty acids, osmolytes, peptides, and proteins) that influence the viability and fertilizing capacity of ejaculated spermatozoa (Mann and Lutwak-Mann, 1981; Shivaji et al, 1990; Yanagimachi, 1994). Thus, the seminal plasmas of a variety of mammalian species contain both factors that prevent inappropriate acrosome reactions and proteins that upon binding to the sperm surface enhance the fertilizing potential of spermatozoa (Killian et al, 1993; Thérien et al, 1997; Rodríguez-Martínez et al, 2005). The concerted action of these regulatory seminal plasma factors modulates the capacitation state of spermatozoa. However, the precise role of most of the seminal plasma proteins on sperm physiology remains obscure. In addition, the effect of seminal plasma on spermatozoa is variable among species, males of the same species, and ejaculates from a single male. The distinct effects exerted by different seminal plasmas on sperm functionality have been in part ascribed to variability of the composition and concentration of some proteins (Maxwell and Johnson, 1999; Centurión et al, 2003).

Spermadhesins are male secretory proteins detected so far in ungulates (pig, cattle, and horse) (Haase et al, 2005). In the pig, this family of proteins consists of 5 members—AQN-1, AQN-3, AWN, PSP-I, and PSP-II (Töpfer-Petersen et al, 1998)—and together they represent over 90% of the total boar seminal plasma proteins (Dostàlovà et al, 1994). The porcine spermadhesin genes are clustered on SCC 14q28–q29 (Haase et al, 2005). Porcine spermadhesins, 110- to 133-residue polypeptides built by a single CUB domain architecture (Romero et al, 1997), are synthesized by the epididymis and accessory glands (Ekhlasi-Hundrieser et al, 2002) and exhibit distinct sperm-coating and ligand-binding capabilities. Sequence variation, glycosylation, and their aggregation state of spermadhesins contribute to their specific pattern of biological activities (Calvete et al, 1993a,b; Dostàlovà et al, 1995a). AQN-1, AQN-3, and AWN coat the sperm surface at ejaculation and display zona pellucida glycoprotein-binding capability and affinity for ß-galactosides and heparin (Sanz et al, 1993; Dostàlovà et al, 1995b; Calvete et al, 1996a,b; Rodríguez-Martínez et al, 1998). These spermadhesins are thought to stabilize the plasma membrane over the acrosomal vesicle and are mainly released from the spermatozoal surface during capacitation (Sanz et al, 1993; Dostàlovà et al, 1994; Calvete et al, 1997). Moreover, AQN-3 has been reported to form part of a sperm motility-inhibitor factor complex (Iwamoto et al, 1995), and AQN-1 and AWN are sperm-associated acrosin-inhibitor acceptor proteins (Sanz et al, 1992). On the other hand, PSP-I and PSP-II are the most abundant boar seminal plasma proteins and form a non-heparin-binding heterodimer (Calvete et al, 1995) of glycosylated spermadhesins (Nimtz et al, 1999). Accumulating evidence points to a role for the PSP-I/PSP-II spermadhesin complex as an exogenous modulator of the uterine immune activity (Leshin et al, 1998; Yang et al, 1998), thus ensuring reproductive success. Hence, PSP-I and PSP-II are immunostimulatory for lymphocyte activity in vitro (Nimtz et al, 1999), and binding of PSP-I to lymphocyte has been demonstrated (Yang et al, 1998). Furthermore, the PSP-I/PSP-II heterodimer and its isolated subunits induce the recruitment of neutrophils into the peritoneal cavity of rats (Assreuy et al, 2002) and pigs (Rodríguez-Martínez et al, 2005). The neutrophil migration-inducing activity of PSP-I/PSP-II, and possibly of the PSP-II subunit, is mediated by the stimulation of resident macrophages, which release a neutrophil chemotactic substance (Assreuy et al, 2002),

whereas PSP-I appears to act directly on neutrophils (Assreuy et al, 2003). The purpose of these proinflammatory and immunostimulatory activities would be to prevent possible infections of the lower reproductive tract and to provide a foreign cell-free uterine environment for the descending early embryos.

Besides their physiological functions in porcine reproduction, the heparin-binding and the non-heparin-binding spermadhesins exert opposite effects on spermatozoa that have been subjected to high dilution to mimic the conditions of sex sorting by flow cytometry (Centurión et al, 2003). Thus, whereas the pooled heparin-binding spermadhesins (AQN-1, AQN-3, and AWN) cause a concentration-dependent sperm membrane damage, the purified non-heparin-binding PSP-I/PSP-II spermadhesin complex contributes to maintaining sperm with high viability, motility, and mitochondrial activity for at least 5 hours at physiological temperature (Centurión et al, 2003). The beneficial effect of the PSP-I/PSP-II heterodimer points to this spermadhesin as a candidate for an additive to improve the viability of highly diluted porcine spermatozoa (ie, flow cytometric sorting for chromosomal sex). In the present study, we sought to dissect the structural basis of the protective effect of boar spermadhesin PSP-I/PSP-II, its isolated PSP-I and PSP-II subunits, and their derived peptidic and glycan fractions on the sperm functionality by a time-course evaluation of the viability, the acrosomal status, the mitochondrial activity, and the motility of highly diluted boar spermatozoa.

Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich Co (Alcobendas, Madrid, Spain) unless otherwise stated.

Preparation of Seminal Plasma

All experiments were performed with the seminal plasma from mature boars that had previously sired offspring. Ejaculates were collected by the gloved-hand method (Larsen, 1986). The seminal plasma was separated from spermatozoa by centrifugation at 3800 x *g* for 15 minutes at room temperature with a Heraeus Sepatech Megafuge (Osterode, Germany). The supernatants were filtered sequentially through 10- and 1.2-µm filters and pooled.

Isolation of the Boar Seminal Plasma PSP-I/PSPII Heterodimer

The PSP-I/PSP-II heterodimer was isolated from the non-heparin-binding fraction of boar seminal plasma by affinity chromatography on a heparin-Sepharose column, equilibrated in 100 mM Tris-HCl; 150 mM NaCl; 5 mM EDTA; and 0.025% sodium azide, pH 7.4, as previously described (Calvete et al, 1995). The identity and purity of the protein was assessed by N-terminal sequence analysis with an Applied Biosystems 472 automated protein sequencer (Applied Biosystems, Langen, Germany) and by MALDI-TOF mass spectrometry with an Applied Biosystems Voyager DE-Pro mass spectrometer (Applied Biosystems) and a saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) as the matrix. Protein concentration was determined spectrophotometrically with the molar absorption coefficient (27 332 M^{-1} cm^{-1}) determined by Menéndez et al (1995) or by amino acid analysis (after sample hydrolysis in 6 M chloride acid (HCl) for 24 hours at 106 °C in evacuated and sealed ampoules) with a Beckman Gold Amino Acid Analyzer (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water and lyophilized.

Isolation of Boar Seminal Plasma PSP-I and PSP-II Subunits

The PSP-I and PSP-II subunits were purified from the heterodimer by reverse-phase high-performance

liquid chromatography (HPLC) on a Lichrocart column (250 x 10 mm, RP-18, 7-µm particle size) (Merck, Germany) eluted at 2 mL/min with a mixture of 0.1% TFA in water (solution A) and 0.1% TFA in acetonitrile (solution B), first isocratically (10% B) for 5 minutes, followed by 30% B for 10 minutes, 45% B for 45 minutes, 70% B for 15 minutes, and 10% B for 15 minutes. The purified PSP-I and PSP-II subunits were dialysed against distilled water and lyophilized. Purity and protein concentration were determined as above.

Isolation of Peptides and Glycopeptides

To separate the peptidic and the glycan moieties, the 100 mg of the PSP-I/PSP-II heterodimer were digested overnight at 37 °C with trypsin by using a 1:100 (wt/wt) enzyme:substrate ratio. Thereafter, the enzyme was inactivated by heating at 100 °C for 2 minutes, and the reaction mixture was lyophilized. Completion of proteolysis was checked by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, reversed-phase HPLC, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Nonglycosylated tryptic peptides were separated from glycopeptides by affinity chromatography on a 5-mL Sepharose-Concanavalin A column (Amersham Biosciences, Uppsala, Sweden) equilibrated and eluted with 20 mM sodium phosphate, pH 7.4, 150 mM NaCl (phosphate-buffered saline [PBS]). ConA-bound glycopeptides were eluted with equilibration buffer containing 100 mM methyl-α-D-mannopyranoside. The glycopeptide fraction was desalted on a C-18 Lichrosphere column (250 x 4 mm, 5-µm particle size) (Merck, Germany), equilibrated, and washed with 0.1% TFA until the absorbance at 214 reached baseline level, followed by elution with 0.1% TFA and 50% acetonitrile. Peptide concentration was determined by amino acid analysis.

Collection of Semen and Evaluation of Sperm Parameters

Sperm-rich fractions from fertile mature boars were collected by gloved-hand method and extended to 30 x 10^6 sperm/mL in Beltsville Thawing Solution (Pursel and Johnson, 1975). Diluted sperm-rich fractions from 3 boars were pooled and spermatozoa were separated from seminal plasma by centrifugation at 1200 x *g* (Megafuge 1.0 R, Heraeus, Germany) during 3 minutes. To avoid sperm membrane damage caused by pipetting spermatozoa directly into dilution medium (Maxwell and Johnson, 1999), the pellet was serially diluted in PBS to a final cell count of 1 x 10^6 sperm/mL and incubated at 38 °C (Steri-Cult 200 incubator, Marietta, Ohio). Samples were taken at 0.5, 2, and 5 hours and analyzed for viability (membrane integrity), acrosomal status, mitochondrial membrane potential, and motility.

Flow Cytometry

Flow cytometric analyses were carried out with a Coulter EPICS XL (Coulter Corporation Inc, Miami, Fla) flow cytometer equipped with standard optics, an argon-ion laser (Cyonics, Coherent, Santa Clara, Calif) performing 15 mW at 488 nm, and the EXPO 2000 software (Coulter Corporation). Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. Nonsperm events (debris) were gated out based on the forward scatter and side scatter dot plot by drawing a region enclosing the cell population of interest. Events with scatter characteristics similar to sperm cells but without reasonable DNA content were also gated out. Forward and sideways light scatter were recorded for a total of 10 000 events per sample. Samples were measured at flow rate of ≈300 cells/s.

Flow Cytometric Assessment of Sperm Viability and Acrosomal Exocytosis

For an accurate estimation of the spermatozoa, the membrane and the acrosome integrity was assessed simultaneously by flow cytometry using the triple staining protocol described by Nagy et al (2003). Briefly, 500 µL of sperm samples (~500 000 cells) were incubated with 50 nM SYBR-14 (using a 100-µM stock solution in DMSO; component A of LIVED/DEAD Sperm Viability Kit; Molecular Probes Europe,

Leiden, The Netherlands), 0.5 µg/mL PE-PNA (peanut agglutinin conjugated with phycoerythrin; Biomeda Corp, Foster City, Calif), and 7.5 µM propidium iodide (PI). The samples were mixed and incubated at 37 °C in dark for 10 minutes before flow cytometric analysis. SYBR-14 was measured with a 525-nm band pass filter, PI was collected with a 620-nm band pass filter, and PE-PNA was detected with a 575-band pass filter. Viable spermatozoa with intact acrosome were defined as those stained only with SYBR-14. Acrosome-reacted spermatozoa were defined as those stained with SYBR-14 and PE-PNA. Spermatozoa stained with PI were classified as dead cells.

Flow Cytometric Assessment of Sperm Mitochondrial Membrane Potential

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzymidazolyl carbocianyne iodide; Molecular Probes Europe), a stain that differentiates cells exhibiting high and low mitochondrial membrane potential (Peña et al, 2003), was used to assess the mitochondrial membrane potential of spermatozoa as described in Martínez-Pastor et al (2004) with slight modifications. A total of 500 µL of sperm samples were incubated in a water bath at 37 °C in dark for 20 minutes with 0.2 µm JC-1, followed by flow cytometric measurement through a 590-nm band pass filter. At low membrane potential JC-1 exists as a green fluorescent monomer, whereas at higher potentials JC-1 forms "J-aggregates" after accumulation in mitochondria that emit a red-orange florescence at 590 nm (Garner and Thomas, 1999; Gravance et al, 2000).

Sperm Motility

Sperm motility was estimated by a computer-assisted sperm motility analysis system with the software Sperm Class Analyzer (Microptic 2002, Barcelona, Spain). Aliquots of 10 µL of semen samples (at 10^6 spermatozoa/mL) were placed in a warm (38 °C) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38 °C) of a Nikon Labophot positive-phase contrast light microscope (Tokyo, Japan) equipped with a 10 x objective and a monochrome video camera (Hitachi CCD model, Chiba, Japan) connected to a personal computer. The program settings were as in Centurión et al (2003). Objective percentage of motile spermatozoa was analyzed in at least 100 spermatozoa per sample.

Effect of Isolated PSP-I and PSP-II and Their Proteolytic Products on Sperm Functions

Diluted spermatozoa (1 x 10^6 sperm/mL PBS) were incubated at 38 °C for 5 hours with 0.75 mg/mL of either 1) native PSP-I, 2) native PSP-II, 3) trypsin-digested PSP-I, 4) trypsin-digested PSP-II, or 5) 1.5 mg/mL of trypsin-digested PSP-I/PSP-II heterodimer. Viability, acrosomal exocytosis, mitochondrial membrane potential, and motility were assessed at 0.5, 2, and 5 hours of incubation. Controls were sperm incubated with 1.5 mg/mL of heterodimer PSP-I/PSP-II or without added proteins.

Effect of the Peptidic and Glycan Fractions of PSP-I/PSP-II on Sperm Functions

Diluted spermatozoa (1 x 10^6 sperm/mL PBS) were incubated at 38 °C for 5 hours with either 1) 1.05 mg/mL of tryptic peptide fraction depleted from glycopeptides by affinity chromatography on ConA-Sepharose (see above) or 2) 0.5 mg/mL of ConA-retained glycopeptides. These amounts of peptides and glycopeptides are equivalent to those released by proteolytic digestion of 1.5 mg/mL of the PSP-I/PSP-II heterodimer. Viability, acrosomal exocytosis, mitochondrial membrane potential, and motility were assessed at 0.5, 2, and 5 hours of incubation. Samples incubated with intact or trypsin-digested PSP-I/PSP-II heterodimer as well as without proteins were used as control.

Dose-Dependent Effect of the Peptidic Fraction of PSP-I/PSP-II on Sperm Functions

Samples of 1 x 10^6 sperm/mL in PBS were incubated at 38 °C for 5 hours with decreasing concentrations of the peptidic fraction of trypsinized PSP-I/PSP-II depleted from glycopeptides by ConA-Sepharose chromatography. The following final concentrations were used: 1.05, 0.52, 0.23, and

0.10 mg/mL, which correspond to 1.5, 0.75, 0.33, and 0.15 mg/mL of the native PSP-I/PSP-II heterodimer. Viability, acrosomal exocytosis, mitochondrial membrane potential, and motility were assessed at 0.5, 2, and 5 hours of incubation. Sperm incubated with 1.5 mg/mL of native heterodimer or without added proteins were used as controls.

For each experiment, the effects of the same batch of lyophilized proteins and their derived proteolytic products were assessed on 4 pools of spermatozoa collected on 4 different days. Each pool was made by mixing spermatozoa from 4 different boars. The same group of 4 boars was used on each day. Duplicate samples were performed for each treatment.

Statistical Analyses

All data editing and statistical analyses were performed in SPSS, version 11.5 (SPSS Inc, Chicago, Ill). Data were analyzed by analysis of variance (ANOVA) by using the Hierarchical Linear Mix Model (MIXED) procedure according to a statistical model including the fixed effect of treatment and of incubation time and the random effect of replicate. To analyze data of sperm viability, motility, mitochondrial activity, and acrosome status, percentages were subjected to arcsine transformation before analysis. When ANOVA revealed a significant effect, values were compared by using the Bonferroni test and were considered to be significant when $P < .05$. Experiments were replicated 4 times.

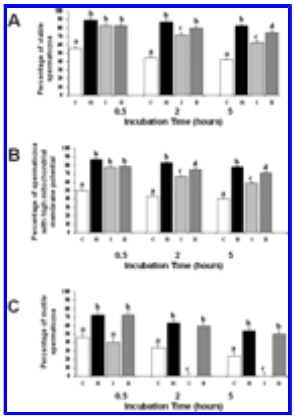

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Figure 1. Effect of the PSP-I and the PSP-II subunits on the viability (sperm with intact acrosome) **(A)**, mitochondrial membrane potential **(B)**, and motility **(C)** of highly diluted boar spermatozoa. Spermatozoa were diluted to 1 x 10^6 sperm/mL phosphate-buffered saline and incubated for 0.5, 2, and 5 hours at 38°C with 1.5 mg/mL of native PSP-I/PSP-II (H), 0.75 mg/mL of isolated PSP-I subunit (I), and 0.75 mg/mL of purified PSP-II subunit (II). Control was incubated in the absence of added protein (c). Viability and acrosomal status were assessed by triple staining (PI/SYBR-14/PE), the mitochondrial membrane potential was determined by using JC-1, and the percentages of motile spermatozoa were evaluated by a computer-assisted motility analysis system. Columns represent the mean ± SEM (error bars) of 4 samples per treatment evaluated in duplicate. Bars per time not sharing a letter are significantly different ($P < .05$).

Results

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Effect of the Isolated PSP-I and PSP-II Subunits on Highly Diluted Spermatozoa

Figure 1A shows the percentage of viable spermatozoa that have been exposed to the PSP-I/PSP-II heterodimer (H) and its isolated subunits, PSP-I (I) and PSP-II (II). In line with previous reports (Centurión et al, 2003, and references therein), in the absence of added proteins, a significant decrease in the viability of the highly diluted control sample was already shown at 0.5 hours compared with spermatozoa exposed to PSP-I/PSP-II heterodimer and its subunits. The dilution-caused decrease in sperm viability of control sperm continued with the incubation time (reaching 38.2% at 5 hours), whereas the native

PSP-I/PSP-II heterodimer and its isolated PSP-I and PSP-II subunits exerted a protective effect on sperm viability (78.3%, 71.6%, and 57.7%, respectively). However, after 2 hours, lower values ($P < .05$) of viability were recorded in samples incubated with PSP-I compared with those containing PSP-I/PSP-II or PSP-II (Figure 1A).

The same trend was observed regarding the percentages of spermatozoa with high mitochondrial membrane potential (Figure 1B). After 5 hours of incubation, the native PSP-I/PSP-II heterodimer, PSP-I, and PSP-II all preserved the mitochondrial activity in 75.5%, 68.1%, and 53.8% of the spermatozoa, though the PSP-I subunit showed a lower value ($P < .05$). For comparison, only 40.1% of spermatozoa retained this activity in the absence of added proteins.

Regardless of incubation time, the percentage of motile spermatozoa was higher ($P < .05$) in spermatozoa that were exposed to the native heterodimer (72.8% at 0.5 hours) or to the PSP-II subunit (75.5% at 0.5 hours) than that of control sperm (46.7% at 0.5 hours) and of spermatozoa incubated with the PSP-I subunit (39.6% at 0.5 hours) (Figure 1C). Moreover, after 2 hours of incubation, PSP-I had a marked detrimental effect on sperm motility, which dropped to undetectable values. This effect was not attributed to a membrane damage because the percentages of acrosome-reacted highly diluted spermatozoa at 0.5, 2, and 5 hours of incubation with the heterodimer or with PSP-I were not significantly different from that of control sperm (Table 1). On the other hand, the exposition of spermatozoa to PSP-II resulted in higher values ($P < .05$) of viable spermatozoa showing a reacted acrosome (Table 1).

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Table 1. *Percentage (mean ± SEM) of viable acrosome-reacted spermatozoa in samples of highly diluted boar spermatozoa (1 x 10^6 sperm/mL) as a function of the incubation time at 38°C and of the presence or absence (control) of native PSP-I/PSP-II and its isolated PSP-I and PSP-II subunits*[*]

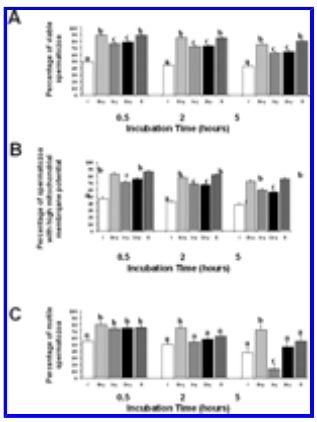

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Figure 2. Effect of trypsin-digested PSP-I (Idig) and PSP-II (IIdig) subunits on the viability **(A)**, mitochondrial membrane potential **(B)**, and motility **(C)** of highly diluted boar spermatozoa. Spermatozoa were diluted to 1 x 10^6 sperm/mL in phosphate-buffered saline and incubated for 0.5, 2, and 5 hours at 38°C with 1.5 mg/mL of intact PSP-I/PSP-II (H), 1.5 mg/mL of trypsin-digested heterodimer (Hdig), 0.75 mg/mL of trypsin-digested PSP-I subunit (Idig), and 0.75 mg/mL of trypsin-digested PSP-II subunit (IIdig). Control samples were incubated in the absence of added protein (c). Viability and acrosomal status, mitochondrial membrane potential, and the percentage of motile spermatozoa were evaluated as in Figure 1. Columns represent the mean ± SEM (error bars) of 4 samples per treatment evaluated in duplicate. Bars per time not sharing a letter are significantly different ($P < .05$).

Effect of Trypsin-Digested PSP-I/PSP-II, PSP-I, and PSP-II on Highly Diluted Spermatozoa

Figure 2A shows the time-course effect of incubating highly diluted boar spermatozoa with trypsin-degradation mixtures of PSP-I/PSP-II (Hdig), PSP-I (Idig), and PSP-II (IIdig). The percentages of viable spermatozoa were significantly higher ($P < .05$) in sperm exposed to trypsin-digested

heterodimer than to either of its trypsin-digested subunits or the control. It is worth nothing that there were no major differences when these results were compared with those obtained with the native proteins, and the same was observed upon analysis of the percentage of spermatozoa displaying high mitochondrial membrane potential (Figure 2B). However, after 2 hours of incubation, the percentage of motile spermatozoa remained higher ($P < .05$) in the samples incubated with trypsin-digested PSP-I/PSP-II heterodimer (72.1%) than in any other condition (Figure 2C). Moreover, trypsin-digested PSP-I subunit exerted a marked negative effect on sperm motility, thus mimicking the effect observed with the native PSP-I subunit (Figure 1C). Similar to what had been noticed when intact proteins were tested, there were no differences ($P > .05$) in the percentages of acrosome-reacted spermatozoa between controls and samples incubated in the presence of intact or trypsin-digested PSP-I/PSP-II and PSP-I subunit (Table 2). However, viable spermatozoa exposed to digested PSP-II subunit showed higher values ($P < .05$) of acrosome-reacted spermatozoa (Table 2).

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Table 2. *Percentage (mean ± SEM) of viable acrosome-reacted spermatozoa in samples of highly diluted boar spermatozoa (1 x 10^6 sperm/ml) as a function of incubation time at 38°C and of the presence or absence of trypsin-digestion mixtures of PSP-I/PSP-II (Hdig), PSP-I (Idig), and PSP-II (IIdig) subunits; sperm samples incubated without added proteins and with native PSP-I/PSP-II represent controls*[*]

Effect of Glycopeptide and Peptide Fractions on Highly Diluted Boar Spermatozoa

The presence in the incubation medium of trypsin-digested PSP-I/PSP-II heterodimer (Hdig), the glyco-peptide fraction of PSP-I/PSP-II (Glyc), and the glycan-depleted tryptic-peptide fraction of PSP-I/PSP-II improved ($P < .05$) the sperm viability compared with the control at all times tested (Figure 3A). However, the percentages of viable spermatozoa were significantly different among samples. Thus, spermatozoa exposed to Hdig or Pept exhibited the highest values ($P < .05$) of viable spermatozoa (72.4% and 82.2% at 5 hours, respectively), which were not different from the percentage of spermatozoa incubated with the intact heterodimer (76.6%). By contrast, spermatozoa incubated with the glycopeptide fraction showed lower values during the 5 hours of incubation (51.3% at 5 hours), though they were significantly higher ($P < .05$) than in the control by about 12%-15%. The same trend was observed in the percentages of spermatozoa showing high mitochondrial membrane potential (Figure 3B).

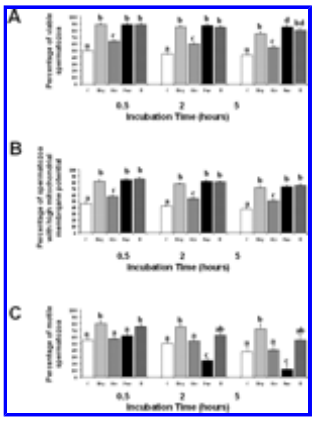

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Figure 3. Effect of trypsin-digested PSP-I/PSP-II heterodimer (Hdig), the affinity-purified peptide (Pept), and the glycopeptide (Glyc) fractions on the viability **(A)**, mitochondrial membrane potential **(B)**, and motility **(C)** of highly diluted (1 x 10^6 sperm/mL) boar spermatozoa. Spermatozoa were incubated for 0.5, 2, and 5 hours at 38°C with 1.5 mg/mL of trypsin-digested PSP-I/PSP-II heterodimer (Hdig), 0.5 mg/mL of glycopeptides (Glyc), and 1.05 mg/mL of tryptic peptides (Pept). Controls were sperm incubated without added proteins (c) and spermatozoa incubated with 1.5 mg/mL of native PSP-I/PSP-II heterodimer (H). Viability and acrosomal status, mitochondrial membrane potential, and the percentage of motile spermatozoa were evaluated as in Figure 1. Columns represent the mean ± SEM (error bars) of 4 samples per treatment evaluated in duplicate. Bars per time not sharing a letter are significantly different ($P < .05$).

At all incubation times, the percentage of motile spermatozoa was significantly higher in samples exposed to either native or trypsin-digested PSP-I/PSP-II heterodimer than in any other condition tested, including the control (Figure 3C). The time-dependent effect of incubating spermatozoa without added proteins (c) and in the presence of the PSP-I/PSP-II glycopeptide fraction (Glyc) were indistinguishable (Figure 3C). On the other hand, the percentage of motile spermatozoa after incubation in the presence of 1.05 mg/mL of the glycan-depleted tryptic-peptide fraction of PSP-I/PSP-II (Pept) markedly decreased with the incubation time from 68.7% at 0.5 hours to 29.4% at 2 hours and 4.1% at 5 hours (Figure 3C).

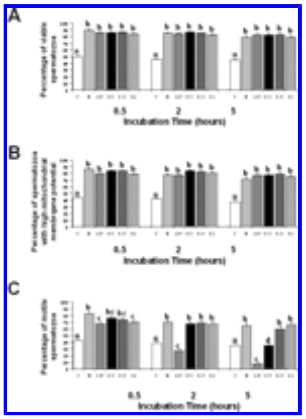

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Figure 4. Effect of decreasing concentrations of the affinity-purified (glycopeptide-depleted) tryptic peptide mixture of the PSP-I/PSP-II heterodimer on the viability **(A)**, mitochondrial membrane potential **(B)**, and motility **(C)** of highly diluted (1 x 10^6 sperm/mL) boar spermatozoa. Spermatozoa were incubated for 0.5, 2, and 5 hours at 38°C with 1.05, 0.52, 0.23, and 0.10 mg/mL of the peptide mixture. Samples incubated with 1.5 mg/mL of PSP-I/PSP-II heterodimer (H) and without added proteins (c) acted as controls. Viability and acrosomal status, mitochondrial membrane potential, and the percentage of motile spermatozoa were evaluated as in Figure 1. Columns represent the mean ± SEM (error bars) of 4 samples per treatment evaluated in duplicate. Bars per time not sharing a letter are significantly different ($P < .05$).

Dose-Dependent Effect of the Peptidic Fraction of PSP-I/PSP-II on Highly Diluted Boar Spermatozoa

Decreasing the concentration of glycan-depleted tryptic-peptide fraction of PSP-I/PSP-II in the incubation medium of diluted spermatozoa from 1.05 to 0.1 mg/mL did not change the percentage of viable spermatozoa, which were always significantly higher (>35%-40%) than in the controls (Figure 4A). The effect produced by different concentrations of peptide backbone on the percentage of spermatozoa with high mitochondrial membrane potential showed the same trend (Figure 4B). However, increasing concentrations of the peptidic fraction had an incubation time-dependent detrimental effect on the motility of the highly diluted spermatozoa (Figure 4C). Thus, sperm motility decreased from 65.8% to 8.5% as the concentration of added peptide fraction increased from 0.1 to 1.05 mg/mL. However, the percentages of acrosome-reacted spermatozoa at 0.5, 2, and 5 hours of incubation remained below 3% in any condition, and no significant differences between groups were detected (data not shown).

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Biotechnological manipulations of semen, such as cooling and deep freezing, in vitro fertilization, or for sexing by flow cytometry or cell-sorting procedures, requires extending the viability of diluted spermatozoa. Sperm extension is linked with the wash away, or high dilution of seminal plasma components (Maxwell and Johnson, 1999), despite the fact that the seminal

plasma contributes to preserving the integrity and the fertilizing potential of sperm and also provides metabolic support to spermatozoa. A detrimental effect on spermatozoa highlighted by an increase of the percentage of cell death, changes in the metabolic activity, and a decrease in their fertilizing activity has been extensively documented (reviewed in Maxwell and Johnson, 1999). Although the composition of diluents has been improved by more-or-less empirical studies, the formulation of species-specific extenders to overcome the detrimental effects of sperm dilution remains a subject of current discussion in reproduction technology (Levis, 2000). Restoring whole seminal plasma has been proposed for improving sperm functionality after dilution (Garner et al, 2001; Caballero et al, 2004). However, the large, inherent variability of seminal plasmas from homologous males as well as between ejaculates from the same male advises against its use (Killian et al, 1993; Zhu et al, 2000; Garner et al, 2001; Caballero et al, 2004). On the other hand, the use of isolated seminal plasma proteins for sperm extension has the advantage of avoiding the inherent variability of the whole seminal plasma. In a previous work, we have shown that different members of the boar spermadhesin family, the purified glycoprotein PSP-I/PSP-II spermadhesin and the pooled heparin-binding spermadhesins exert antagonistic effects on the functionality of highly diluted boar spermatozoa (Centurión et al, 2003). The finding that PSP-I/PSP-II contributes to maintaining sperm with high viability, motility, and mitochondrial activity for at least 5 hours at physiological temperature points to its potential use as an additive for sperm preservation (Centurión et al, 2003) and prompted us to investigate the nature of the sperm protecting epitopes.

First, we studied the effects of the isolated PSP-I and PSP-II subunits on sperm functions. The PSP-II subunit mimicked the effect of the native heterodimer on maintaining sperm viable, motile, and with a high mitochondrial membrane potential (Figure 1). The effect of the PSP-I subunit was beneficial, though less pronounced than that of PSP-II on sperm viability and mitochondrial potential. However, it almost completely abolished sperm motility after 2 hours of incubation (Figure 1). Whether PSP-I might be affecting the glycolytic pathway of the spermatozoa is as yet unknown. This detrimental effect of PSP-I follows a similar time course and magnitude to the previously noticed sperm-immobilizing activity exerted by the pooled heparin-binding spermadhesins (Centurión et al, 2003). PSP-I is a glycosylated spermadhesin, and glycosylation indirectly modulates its ligand-binding properties. Thus, mannose-rich PSP-I glycoforms have been reported in the heparin-binding fraction of spermadhesins (Calvete et al, 1993b), whereas complex-type PSP-I glycoforms specifically associate with PSP-II into a noncovalent heterodimer (Calvete et al, 1995; Nimtz et al, 1999). The fact that the PSP-I glycoprotein isolated from PSP-I/PSP-II abolished the motility of highly diluted spermatozoa whereas the native heterodimer did not strongly suggested that this activity might be suppressed upon dimerization. Other binding activities impaired by complex formation are the heparin-binding capability and the mannose-6-phosphate recognition by PSP-II (Solis et al, 1998). Taken together, these results suggest a mechanism for the modulation of the ligand-binding properties of PSP-I and PSP-II.

To further dissect the effects of the PSP-I/PSP-II subunits on sperm functions, the native heterodimer and the isolated subunit were degraded with trypsin and the resulting peptide mixtures were checked for their activities on sperm parameters. The protective effect of the PSP-II on viability, mitochondrial membrane potential, and motility of highly diluted spermatozoa were not abolished by trypsin digestion, indicating that the conformation of the active epitope or epitopes was not destroyed by proteolysis. Similar results were observed when the PSP-I/PSP-II digestion mixture was analyzed. Moreover, the biological activity of the heterodimer was amplified after trypsin digestion compared with the native protein. In contrast, the negative effect of PSP-I on sperm motility was clearly attenuated. Hence, we may conclude that degradation of the native PSP-I/PSP-II exposes active epitopes, which remained hidden in the quaternary structure of the heterodimer or in the tertiary structure of its constituent subunits.

To evaluate the activity of the peptide and the glycan moieties of PSP-I/PSP-II, the tryptic peptide mixture of the heterodimer was fractionated by affinity chromatography on ConA-Sepharose. The nonbound (nonglycosylated peptides) and the bound (glycopeptides) fractions were used at 1.05 mg/mL and 0.5 mg/mL, respectively, which correspond to their calculated concentrations for a 1.5 mg/mL solution of native PSP-I/PSP-II heterodimer. This PSP-I/PSP-II concentration represents the optimal protective dose determined in previously reported experiments (Centurión et al, 2003). The data displayed in Figure 3 show that the peptide fraction retained the biological activity of PSP-I/PSP-II on the viability and mitochondrial activity of highly diluted boar spermatozoa, whereas the glycopeptide fraction exhibited a light positive effect, which was not different from the control. In addition, the glycopeptide fraction had no effect on sperm motility, whereas the peptide fraction abolished in a time-dependent manner the motility of viable spermatozoa (Figure 3C). This net detrimental activity may represent a composite of actions of the motility-impairing activities of native and trypsinized PSP-I and the positive effect of native and trypsin-degraded PSP-II (Figures 1C and 2C).

The deleterious effect of the tryptic peptide fraction of the PSP-I/PSP-II heterodimer followed a marked concentration-dependent pattern. Thus, decreasing the total peptide concentration from 1.05 to 0.1 mg/mL restored sperm motility to the same level observed with the intact heterodimer, and this effect lasted for at least 5 hours.

In conclusion, the subunits of the PSP-I/PSP-II heterodimeric disintegrin exert different activities on sperm functions. The beneficial effect of the native PSP-I/PSP-II on the functionality of highly diluted boar spermatozoa is largely preserved in its isolated PSP-II subunit and does not appear to require its glycan moiety.

Acknowledgments

The authors thank Dr William V. Holt (Institute of Zoology, Zoological Society of London, London, United Kingdom) for reading the manuscript.

Footnotes

Supported by grants AGL2001-0471, RZ01-019, and BFU2004-01432 from the Ministerio de Educación y Ciencia, Madrid, Spain.

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