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The trefoil domain of porcine zona pellucida: a sperm receptor?

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Abstract

Trefoil domains are protein structures characterized by six cysteines, which form a three-loop structure by disulfide bridges. Between the loops, a hydrophobic groove builds a binding pocket for carbohydrates or proteins. The porcine zona pellucida protein ZP4 contains such a trefoil domain (TFD); therefore we suggested a potential sperm receptor function. Sperm-egg interaction is known to be a multi-step event; thus the aim of the study was to analyze the interaction of porcine TFD with boar sperm cells at several levels which reflect the most important steps of the fertilization cascade. We tested by Far Western blot and surface plasmon resonance whether a synthetic porcine TFD (pTFD) was able to interact with sperm cell lysates and by immunocytochemistry if it is able to bind sperm cell membranes. No interaction could be shown, although we previously demonstrated the three-dimensional folding of the synthetic pTFD by a trypsin resistance assay. Furthermore, we determined the ability of pTFD to affect sperm capacitation or to induce the acrosome reaction. An influence was not measurable for capacitation or for the induction of acrosome reaction. We also investigated the influence of porcine TFD on sperm-ZP binding. In contrast to an anti-pZP antibody, the pTFD was not able to inhibit sperm binding to oocytes. In summary, the sperm receptor function of TFD within pig zona pellucida could not be proven. Further research is necessary to identify the function of TFD within zona pellucida; a structural role during oviduct passage is suggested.

Keywords: acrosome reaction, fertilization cascade, pig, sperm proteins.

Introduction

Fertilization comprises a set of complex gamete interaction processes, which is still a puzzle to be solved. Several structural components on the surface of mammalian oocytes and spermatozoa have been identified as sperm-egg receptors. Based on these findings, it has become clear that gamete recognition in mammals involves a multiplicity of carbohydrate and/or protein based binding mechanisms (Shur, 2008).

It is generally accepted that the initial sperm-egg binding step is mediated by an interaction between molecules of the sperm plasma membrane and molecules of the egg envelope – the zona pellucida (ZP). The ZP consists of three to four glycoproteins: ZP1, ZP2, ZP3 and ZP4 (Conner *et al.*, 2005), which play an essential role within the fertilization cascade. Although the DNA and amino acid sequences of zona pellucida proteins are quite conserved among mammalian species, the sperm receptor function is species-specific implemented. In that context, it has been shown that the carbohydrate chains of glycosylated ZP proteins seem to be responsible for the conspecific first interaction with sperm cells. After replacement of murine ZP2 and ZP3 by human ZP2 and ZP3 constructs in mice, the binding of exclusively conspecific murine, but not human spermatozoa, was observed (Rankin *et al.*, 1998, 2003).

Conversely, it is also evident that sperm cells interact with ZP proteins. Apart from the initial sperm contact to the ZP, which enables the acrosome reaction, the so-called secondary binding (Bleil and Wassarman, 1983) mediates penetration of sperm cells through the ZP. This essential step of gamete interaction is blocked among other changes by the release of protease from cortical granules which occurs when the first sperm cell had fused to the oocyte (Bleil and Wassarman, 1980a, b). Mouse eggs containing human ZP2 loose the ability to block polyspermy after fertilization (Rankin *et al.*, 2003). This is explained by a failure of human ZP2 to undergo proteolytic cleavage by the protease released from murine cortical granules. Specific protein structures within the three-dimensional ZP are responsible for secondary sperm binding. Hoodbhoy and Dean (2004) suggested that sperm cells recognize a supramolecular scaffold rather than specific glycan chains on the ZP. In this regard, it is important to mention that carbohydrate-based reagents rarely inhibit sperm-egg binding completely, suggesting that some binding is independent of carbohydrate-based mechanisms (for review: Shur, 2008).

Whereas the role of ZP3 and ZP2 in primary and secondary sperm binding has been comprehensively investigated in mice, the knowledge about function of ZP components in other species is limited. It seems that in other species different ZPs may be involved in binding processes. Yurewicz *et al.* (1998) have shown a hetero-oligomerization-dependent binding of pig oocyte ZP4 and ZP3 to boar sperm membrane vesicles. Furthermore, Chakravarty *et al.* (2008) delineated the influence of glycosylation of human ZP glycoproteins for their binding to spermatozoa. They identified a

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glycosylation-independent ZP3 and ZP4 binding to capacitated spermatozoa; ZP2 was only able to bind acrosome reacted sperm cells. Additionally Chiu *et al.* (2008) demonstrated the influence of human ZP3 and ZP4 on the acrosome reaction and human spermatozoa ZP binding.

The current study focused on a special structure of ZP4. An antibody raised in rabbits against feline ZP was not only able to inhibit in-vitro-fertilization (Jewgenow *et al*., 2000), but also recognized, among other epitopes, the first loop of a trefoil-domain (TFD) of feline ZP4 (Ringleb *et. al.*, 2004; Braun *et al*., 2009). Govindt *et al.* (2000) showed that antibodies directed against an epitope located proximate to the first loop of this domain inhibited sperm-egg binding in bonnet monkey. Trefoil-domains are protein structures characterized by six cysteines which form a characteristic three-loop structure by three disulfide bridges in a 1-5, 2-4, 3-6 combination. Typical representatives of this protein family are trefoil factors which form a hydrophobic groove between peptide loops, a potential binding pocket for carbohydrate residues or proteins (Petersen *et al*., 1996; Polshakov *et al.*, 1997; Lemercinier *et al.*, 2001; Thim and May, 2005). Therefore, we suggest that the antigen TFD of the ZP1/ZP4 (Bork, 1993) represents a further potential receptor for carbohydrate as well as for protein-mediated sperm binding.

Against the background of designing a ZP-based contraceptive vaccine for wild pigs, we investigated the role of TFD in the porcine system. For this purpose, the porcine TFD peptide sequence was identified and an artificial peptide (pTFD) was synthesized. We tested whether pTFD was able to interact with sperm cell lysates, to bind sperm cell membranes, to influence sperm capacitation or to induce the acrosome reaction. We also investigated the influence of pTFD on sperm-ZP binding.

Materials and Methods

All chemicals were obtained from Sigma-Aldrich GmbH (Steinheim, Germany) unless stated otherwise and were of the highest purity available.

Trefoil domain peptide and anti-pZP antibody

A porcine trefoil domain was synthesized (aa 140-183) by Biosynthan GmbH (Berlin, Germany) and labeled with biotin at the C-terminal lysine (pTFD-Bio). Antibody against porcine ZP (anti-pZP) was produced in rabbit as previously described (Jewgenow *et al.*, 2000).

Trypsin assay to identify peptide folding

Porcine TFD-Bio (0.375 mg/ml) was digested in 0.01 mg/ml trypsin in the presence or absence of 110 mM DTT (1,4-dithiohreitol, Carl Roth GmbH, Karlsruhe, Germany) at 37°C in PBS for up to 1 h. Trypsin was omitted in the control group. In order to assess protein stability, aliquots were taken at different time points (0, 5, 10, 30 and 60 min) and analyzed by SDS-PAGE (Laemmli, 1970). For this, aliquots were mixed with 1/10 volume 100 mM PMSF (phenylmethanesulfonyl fluoride) and SDS-PAGE-sample buffer [final concentration: 50 mM Tris-HCl pH 6.8, 1% SDS (w/v), 12.5% glycerol (v/v), 0.01% bromphenol blue (w/v), 125 mM DTT] and boiled for 5 min at 95°C. These samples were subjected to a 20% SDS-PAGE (Laemmli, 1970) followed by Coomassie blue staining of the gel [0.23% (w/v) Coomassie brilliant blue R-250 in 45% methanol, 10% acetic acid and 45% H_2O (all v/v)].

Semen collection

Landrace boar semen (n = 3) was collected by ejaculation at boar stations of the Land Brandenburg. The semen was diluted with BTS (Beltsville thawing solution, Minitüb GmbH, Tiefenbach, Germany) and tubes containing liquid-preserved semen were maintained at 16°C until use. Storage was not longer than 48 h.

Capacitation and acrosome reaction of boar spermatozoa

Boar spermatozoa capacitation and acrosome reaction was performed according to Kurz *et al.* (2005). Briefly, liquid-preserved boar sperm cells in BTS were incubated for 10 min at 38.5°C and washed two times with capacitation medium (albumin-lactate-pyruvate modified Tyrode medium; co – control sperm cells). After washing, capacitation was undertaken in the presence of 1.18 U/ml heparin and 2 mM $CaCl_2$ at 38.5°C for 45 min (ca – capacitated sperm cells). To test the effect of pTFD-Bio during capacitation on sperm cell responsiveness to calcium ionophore, 50 µg/ml pTFD-Bio was added or heparin was displaced by it. The acrosome reaction was induced with 3 µM Ca^{2+}-ionophore A23187 (per 2 x 10^7 sperm cells) at 38.5°C for 30 min (ar – acrosome reacted sperm cells). In order to analyze whether the peptide was capable to induce the acrosome reaction, 50 µg/ml pTFD-Bio was added to capacitated boar sperm cells instead of calcium ionophore.

Isolation of sperm cell proteins

Sperm samples were centrifuged at 700 x g for 8 min at room temperature (RT). The supernatants were supplemented with 10% (v/v) glycerol (supernatants samples: coSu, caSu, arSu) and the sperm pellets were washed two times in HEPES-buffer A (5 mM HEPES, 150 mM NaCl, pH 7.4) and stored at -20°C until cell lysis. After thawing, the sperm pellets were homogenized in HEPES-buffer A containing 0.5% (v/v) Triton X-100 and 10% (v/v) glycerol using an eppi-

pistill on ice for 2 min. Samples were centrifuged at 1300 x g for 5 min to remove insoluble constituents (supernatant = sperm lysate samples: coSp, caSp, arSp). Protein content of all samples was determined by BCA protein assay as described before (Smith *et al.*, 1987).

Detection of acrosome reaction by flow-cytometry

Sperm cells were fixed in 0.125% (v/v) formaldehyde in PBS (final concentration) and stained with 2.5 µg/ml FITC-PSA (fluorescently labeled lectin from *Pisum sativum*) for 10 min, as marker for acrosomal contents followed by incubation with 5 µg/ml propidium iodide (PI, Invitrogen, Karlsruhe, Germany) for 5 min, as marker for dead sperm cells. After incubation in the dark, 10 µl of the sperm suspension was added to 2 ml PBS and 15,000 cells were analyzed by flow-cytometry (Partec GmbH, Münster, Germany) using a 400 mW argon laser (Ex 488 nm), a 515-560 band-pass for FITC and a 620 nm long-pass filter for PI.

Surface plasmon resonance

Surface plasmon resonance (SPR) was used to identify sperm lysate proteins interaction with pTFD-Bio. This technique uses special chips coated with biomolecules to determine biomolecular interactions in real-time. SPR biosensor (Biacore 2000, GE Healthcare, München, Germany) detects changes in the aqueous layer close to the sensor chip by measuring the refractive index expressed as resonance signals (Torreri *et al.*, 2005). This method is highly sensitive and does not depend on labeling. The SPR biosensor unit was equipped with a streptavidin-coated sensor chip SA (GE Healthcare, München, Germany). In a first step, pTFD-Bio and a control peptide were bound to the chip in two different flow cells. Interaction between the streptavidin-chip and the biotinylated peptides was evident by achieving stable binding signals (not shown). After addition of prospective binding partners, the specific interaction with pTFD-Bio was detected by measuring the difference in signal between pTFD-Bio flow cell and the control peptide flow cell. An increasing signal during incubation phase was interpreted as specific binding. The following samples were tested according to their surface resonance with pTFD-Bio: coSp, caSp, arSp, anti-pZP. All samples were injected at a flow rate of 5 µl/min in HEPES-buffer A. Bound samples were removed with glycine pH 2.5, pH 2.0 and pH 1.5 until baseline was reached. Sperm samples were analyzed in the presence of a NSB reducer (GE Healthcare, München) to minimize unspecific binding.

Far Western blot of sperm samples

Sperm lysates and cell free supernatants (10 µg coSu, 20 µg caSu, 20 µg arSu, 3.75 µg coSp, 3.75 µg caSp, 3.75 µg arSp per lane) and pTFD-Bio (0.7 µg) were separated on 12% SDS-PAGE (Mini Protean III; Bio-Rad, München, Germany) and transferred to a nitrocellulose membrane (0.2 µm, Protran BA83, GE Healthcare, München, Germany) using a semi-dry blotting methodology (1 h, 1.2 mA/ cm^2 membrane). Following transfer, membranes were incubated in blocking solution [1% (w/v) BSA, 1% (w/v) milk powder, 0.05% (v/v) Tween-20 PBS] for 1 h at RT followed by an incubation with pTFD-Bio (20 µg/ml in PBS) or PBS (as control) for 10 min. Membranes were subjected to a short fixation in 3.7% (v/v) formaldehyde in PBS for 10 min at RT and washing steps before and after the fixation (in PBS at RT, in each case three times for 1 min) before they were incubated with anti-pZP (1:5.000) or streptavidin-peroxidase (130 ng/ml) at 4°C overnight. After washing [0.2% (v/v) Tween-20 in PBS, three times 10 min at RT], specific binding of anti-pZP was detected by using a peroxidase-conjugated goat anti-rabbit IgG secondary antibody diluted 1:20000 (Sigma, A0545). Visualization of POD-antibody and streptavidin-POD binding was achieved with the ECL advanced reagent (GE Healthcare, München, Germany).

Binding of TFD to sperm cells (Immunocytochemistry)

Smears were produced from boar sperm cell samples before and after induction of the acrosome reaction. After 16 h air-drying, the smears were fixed in 0.1% (v/v) formalin and stored at -80°C until use. Slides were incubated in PBS containing 50 µg/ml pTFD-Bio or PBS for 3 h at RT and washed afterwards three times for 10 min in PBS. After incubation in blocking buffer [3% (w/v) BSA, 0.1% (v/v) Tween-20 in PBS] for 3 h at RT all smears were treated with 50 µg/ml Streptavidin-FITC for 1 h at RT in the dark. Prospective binding sites of pTFD-Bio were analyzed under fluorescence excitation using a DMLB Leica Microscope (Wetzlar, Germany).

Boar sperm binding to pig zona pellucida

Slaughterhouse-derived porcine ovaries were transported to the laboratory in PBS supplemented with 10 ml/l antibiotic solution (comprising 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml) at 30°C. Whole ovaries were sliced for oocyte collection in PBS containing 1 mM EDTA (ethylenediamine-tetraacetic acid). Obtained pig oocytes were pooled according to quality as intact compact cumulus- class I or II, atretic oocytes, nude oocytes, and intact cumulus oocyte complexes were selected, denuded through pipetting in PBS, and then washed in PBS. After washing, groups of 100–200 oocytes were subjected to a very small amount (<10 µl) of media and transferred to 500 µl freezing solution composed of 10% DMSO (dimethyl sulfoxide) in FCS (fetal calf

serum), stored overnight at -70–80°C and then plunged into liquid N_2 (Lynham and Harrison, 1998).

Cryostored pig oocytes were allowed to thaw at RT and then washed three times in 4-well plates containing 500 µl HEPES-buffer B (20 mM HEPES adjusted to pH 7.5 with NaOH) containing 2 mM EDTA, 1 mg/ml PVA (polyvinyl alcohol), 1 mg/ml PVP (polyvinylpyrrolidone) and 0.055 mg/ml gentamicin. Afterwards, oocytes were subjected to three washes in 500 µl HEPES-buffer B. The duration of each wash was 15 min. Oocytes were transferred to insemination drops containing 400 µl of *in vitro* fertilization (IVF) medium (TALP solution containing 10 mg/ml heparin) and 1 x 10^5 motile boar spermatozoa per ml, which were pre-incubated for 1 h with pTFD-Bio (0.1 mg/ml). After 18–20 h of co-incubation at 39°C under an atmosphere of 5% CO_2, oocytes were washed three times in DPBS (Dulbecco´s PBS) containing 3 mg/ml BSA to remove loosely attached spermatozoa and finally placed into DPBS supplemented with 4% (w/v) paraformaldehyde and 0.02% (v/v) Triton-X100 for 45–60 min at 39°C. Oocytes were washed twice in DPBS and stained with 10 mg/ml Hoechst 33258 for 45 min, mounted on slides and the number of attached sperm heads was counted under fluorescence microscope. Oocytes pre-treated with anti-pZP serum diluted at 1:20 were also used as a positive control for inhibition of sperm binding. Every experiment was replicated five times.

Statistical analyses

For statistical analysis, means (±SEM) were compared by paired *t*-test (acrosome reaction) or non-parametric Mann–Whitney test (sperm binding test) with a $P < 0.05$ considered significant. Statistical procedures were performed with the software program Instat Version 3 (Graphpad Software Inc.).

Results

Trypsin assay to identify peptide folding

Fig. 1 represents trypsin digestion of pTFD-Bio. Samples were subjected to SDS-PAGE to visualize the influence of trypsin. Digestion of peptides is evident by a quickly vanishing band, which is visible after adding the reducing substance DTT to the TFD. In contrast, under non-reducing conditions pTFD-Bio was not digested by trypsin for at least 30 min. This is an indication that the synthesized peptide expresses the three-dimensional folding together with the suggested protease resistance – a property known as other trefoil domain containing proteins.

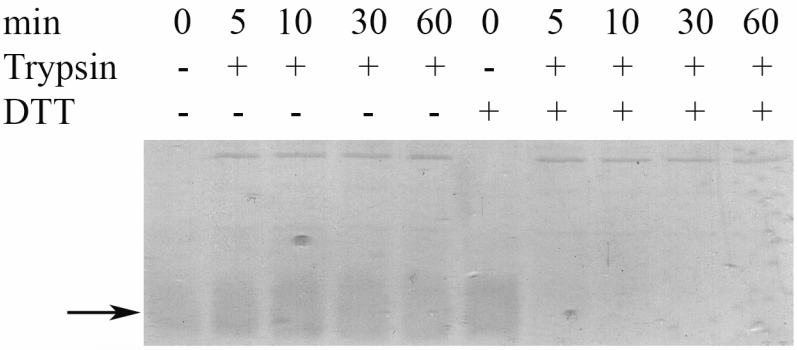
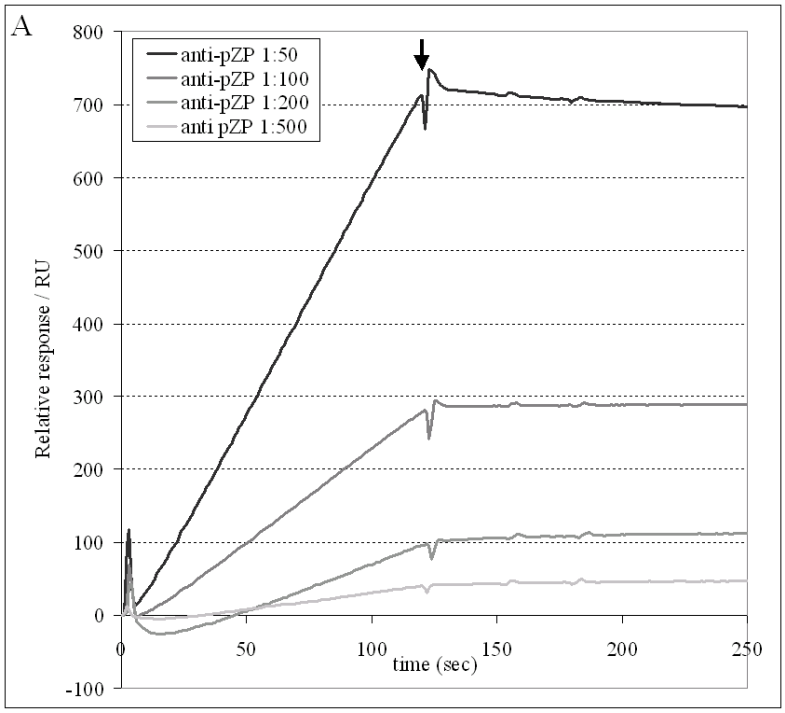


Figure 1. Stability of pTFD-Bio against trypsin digestion. Peptide was incubated with trypsin over 60 min at 37°C in the presence or absence of 110 mM DTT. Samples were evaluated at different time points by SDS-PAGE, followed by Coomassie staining. The loss of pTFD-Bio signals (arrow) over time denotes the digestion of the peptide by trypsin.

Interaction of sperm cell lysates with TFD

Surface plasmon resonance results are shown in Fig. 2. Addition of anti-pZP to the biacore system resulted in a concentration dependent response, indicating an interaction between the ZP antibody and the synthesized peptide pTFD-Bio (Fig. 2A). In contrast, addition of sperm lysates or acrosome reaction supernatant to the detection system did not induce any biomolecular interaction as indicated by the lack of response over time as shown in Fig. 2B. The small peaks at the beginning and end of injection phase (arrows) are explained by a time lag until the sample reaches the two different flow cells.

Far Western blot analysis (Fig. 3) also failed to demonstrate an interaction between sperm samples and the pTFD-Bio. No signals were found in lines 1–6, representing sperm cells, extracts and cell supernatants. Only in line 7, where pTFD-Bio was subjected to PAGE, a prominent signal was evident after visualization with ECL.



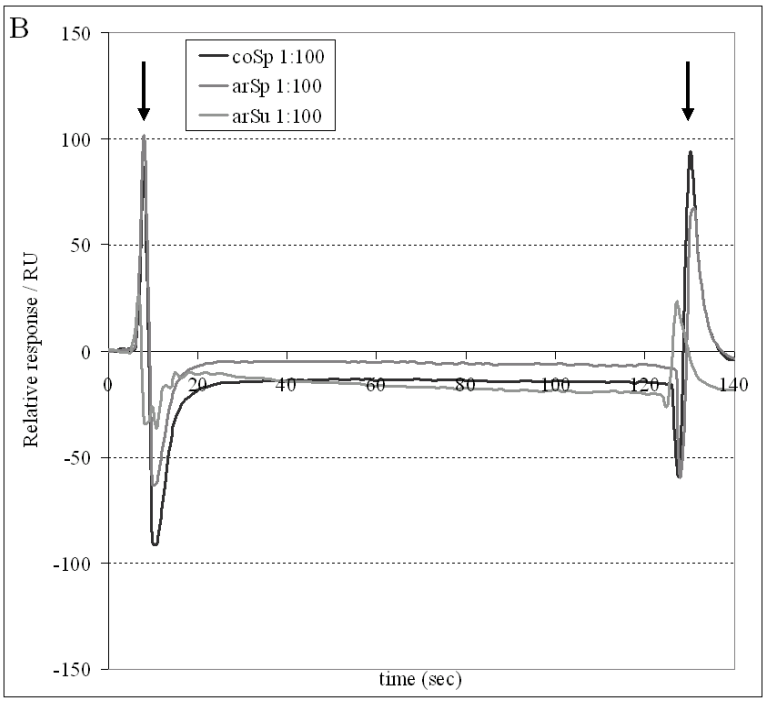


Figure 2. Binding of anti-pZP (A) and boar sperm samples (B) to a pTFD-Bio coated chip (Biacore Sensor chip SA) determined by surface plasmon resonance.
A: The relative response units increase concentration-dependent after injection of anti-pZP. The arrow marked the end of the binding phase.
B: Different sperm samples were injected to the same system (1:100 dilutions). The relative response was not reacting on the injection of the samples, indicating a missing specific binding reaction with the pTFD-Bio. coSp: control sperm lysate, arSp: lysate of acrosome reacted sperm cells, arSu: supernatant of acrosome reacted sperms. The time area between the two arrows marked the incubation phase.

Binding of TFD to sperm cells

Incubation of sperm smears with pTFD-Bio did not result in any fluorescence signal on the sperm cell surface (pictures not shown).

Influence of TFD on acrosome reaction

The effect of pTFD on *in vitro* capacitation and acrosome reaction was investigated to determine the role of TFD on sperm function. Flow-cytometric analysis revealed that untreated liquid-preserved samples had about 90% of viable sperm cells as shown in Table 1. The percentage of FITC-PSA-positive sperm cells was negligible. Capacitation was accompanied by a loss of viability, and a non-significant tendency to an increased exposition of acrosomal contents detected by FITC-PSA. The addition of calcium ionophore A23187 to capacitated spermatozoa increased ($P \leq 0.03$) FITC-PSA-positive cells indicative of acrosome reacted spermatozoa. In contrast to calcium ionophore, pTFD-Bio was not able to induce acrosome reaction in capacitated boar sperm cells. Moreover, the presence of pTFD-Bio during capacitation had no effect on the extent of the ionophore induced acrosome reaction (data not shown).

Table 1. Influence of calcium ionophore (A23187) and pTFD-Bio on boar sperm cell acrosome reaction indicated by PSA labeling.

	Viable sperm cells (%) (PI-negative)	Viable acrosome reacted sperm cells (%) (PI-negative, PSA-positive)	Total acrosome reacted sperm cells (%) (PSA-positive)
*Liquid-preserved sperm cells (co)	90.7 ± 4.6[a]	0.6 ± 0.6[a]	2.4 ± 3.2[a]
Capacitated sperm cells (ca)	54.7 ± 14.0[b]	5.8 ± 3.3	15.1 ± 4.5
Induction of acrosome reaction with:			
A23187 ionophore (ar)	43.4 ± 11.3[b]	12.6 ± 4.9[b]	31.0 ± 9.3[b]
pTFD-Bio	49.8 ± 11.5[b]	4.4 ± 0.9	12.5 ± 3.4
Control medium	41.1 ± 9.5[b]	5.4 ± 2.3	16.3 ± 7.5

*Liquid-preserved sperm cells from three different boars (n = 3) capacitated in the presence of heparin and $CaCl_2$. Acrosome reaction was induced by addition of A23187 or pTFD-Bio. Usually the acrosome reaction is attributed to viable sperm cells. Since acrosome reacted sperm cells rapidly loose their viability *in vitro*, the total number of PSA-positive sperm cells is also given.

[a, b] Different superscripts characterize significant differences within one category of cells (paired t-test, $P \leq 0.03$).

Influence of TFD on sperm binding to oocytes

Pre-incubation of sperm cells with pTFD-Bio had no effect on the number of bound spermatozoa (26.3 ± 3.5 sperm cells per oocyte, n = 65) as compared to the control (22.8 ± 1.7 sperm cells per oocyte, n = 62). Conversely, treatment of oocytes with anti-pZP serum led to a reduction ($P < 0.01$) in sperm binding (0.8 ± 2.4 sperm cells per oocyte, n = 58).

Discussion

The aim of this study was to demonstrate an interaction between boar sperm cells and a synthetically produced porcine ZP trefoil domain. Sperm-egg interaction is known to be a multi-step event; therefore we analyzed the interaction of porcine TFD at several levels, which represent important steps in the fertilization cascade (Bleil and Wassarman, 1983). Although several techniques were applied, we failed to demonstrate a sperm receptor function of pTFD.

One reason for this lack of sperm receptor function could be the incorrect three-dimensional folding of the synthesized peptide. However, we demonstrated the proteolytic resistance of the synthetic peptide, a characteristic property of trefoil factors (Thim and May, 2005). The synthetically produced pTFD-Bio was resistant to trypsin treatment for a prolonged period and lost this property under reducing conditions (Fig. 1). Therefore, it is believed that the trefoil domain was folded in a natural way. Moreover the biotin label at the end of the peptide did not interfere with its molecular structure but was very useful for applying several immunological techniques which were based on streptavidin-POD detection. Antibodies raised against native porcine ZP (Jewgenow *et al.*, 2000) were able to recognize this synthesized peptide (Fig. 2A), another indication that the peptide was folded in the same way, as in the native ZP.

Our first attempt to prove receptor function was a direct binding study of pTFD to boar sperm cell membranes either before or after acrosome reaction.

Therefore, both lysates of sperm cells and supernatants were analyzed according to a prospective binding capacity (Far Western blot) because it is very likely that acrosome content will accumulate in the supernatant of boar sperm cells after ionophore induced acrosome reaction. Despite producing many other proteins (Stein *et al.*, 2006) the acrosome vesicle contains (pro)acrosin (Howes and Jones, 2002) which plays an important role in sperm binding to and penetration of ZP during fertilization. An interaction of pTFD-Bio with acrosome content would imply a role within secondary sperm binding.

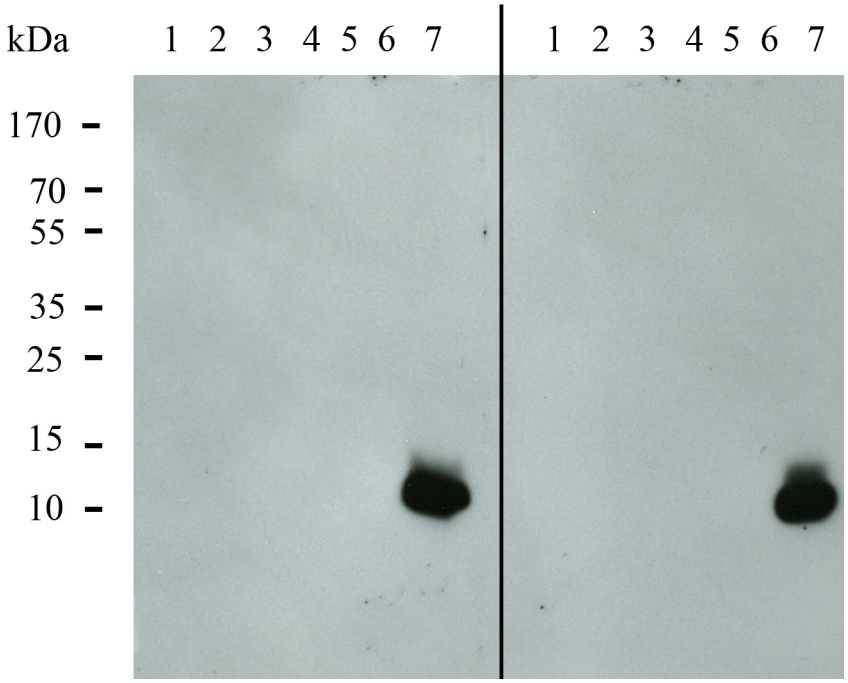


Figure 3. Far Western blot analysis of boar sperm samples. After transferring of separated proteins from a SDS-PAGE to a nitrocellulose membrane and blocking this membrane, the left side of the membrane was incubated in PBS; the right side was incubated in 20 µg/ml pTFD-Bio to recognize binding of TFD to sperm proteins. TFD-Bio was detected by incubation with streptavidin-peroxidase and visualized with ECL. The following samples were applied to the lanes: 1: coSp, 2: caSp, 3: arSp, 4: coSu, 5: caSu, 6: arSu, 7: pTFD-Bio. Sp = sperm lysate; Su: supernatant; co: control; ca: after capacitation; ar: after acrosome reaction.

Potential sperm receptors for ZP proteins, including the TFD domain, might also be located within the sperm cell membranes, either plasma or inner acrosomal membranes. Thus, sperm cell lysis was performed by Triton-X100. Many authors, including Shetty *et al.* (2001), have demonstrated that mild extraction with Triton X-100 can be used for solubilization of membrane and acrosomal proteins. Thus, absence of binding of sperm proteins may indicate that a potential receptor was not found within this lysate. In addition, sperm extracts have to be dissolved in SDS-PAGE sample buffer, which contains DTT – a reducing substance. Therefore, within the SDS-PAGE the sperm proteins lose their tertiary structure. This may be the reason for the disruption of prospective binding sites. Nevertheless, western blot analysis has been proven to be helpful in analyzing sperm-egg binding partners. For example, Howes *et al.* (2001) demonstrated the binding of mouse iodide-marked ZPs to recombinant proacrosin that was separated by SDS-PAGE and transferred to membranes. Kadam *et al.* (2006) identified an oviductal glycoprotein-binding protein partner on sperm cells by Far Western blot.

In order to avoid such structural effects, we applied a new and sensitive method to analyze interactions of biomolecules in real-time: surface plasmon resonance. The main advantages of this approach are that native samples are only required in very small amounts and no labeling is necessary. Furthermore, the method is able to determine the association and dissociation rates of receptors and their ligands (Berggård *et al.*, 2007). Thomas *et al.* (2003) demonstrated an interaction of bovine seminal plasma protein, PDC-109, with lipids by using surface plasmon

resonance. Whereas Maegawa *et al.* (2002) applied this method for analyses of sperm extract binding to carbohydrates. Although the surface plasmon resonance could determine the protein interaction between pTFD-Bio and anti-pZP antibody (Fig. 2A), we failed to demonstrate an interaction of sperm samples with pTFD-Bio (Fig. 2B).

Finally, structural and functional intact sperm cells were analyzed for interaction with pTFD. This was performed by immunocytochemistry using fluorescence labeled streptavidin in accordance with the methods described by Moos *et al.* (1993) and Leong *et al.* (1999). They detected sperm protein on the surface of air-dried and fixed sperm cells (Moos *et al.*, 1993). Functionally intact sperm cells are not only viable but also able to perform acrosome reaction and bind and penetrate the intact ZP. All these essential functions of boar sperm cell were not influenced by the co-incubation with pTFD as shown by flow-cytometry and *in vitro* sperm binding assay to porcine ZP. The concentrations we used in our trials (0.2 to 20 µM pTFD) were at the same level or actually higher than concentrations of ZP-peptides or ZP-proteins applied in previous studies (Bausek *et al*., 2004; Gahlay *et al.* 2005; Hinsch *et al.* 2005). All our tests lead to an identical result, that the pTFD is not involved in the sperm-egg binding. Anyway we found it important to present our approach, because all used test systems include advantages and disadvantages in characterizing the multi-step process of fertilization.

Disproving our original hypothesis, the question about the functional role of TFD within the ZP still needs to be answered. There are numerous indications that TFD plays a structural role. The protease resistance might be important for the zonal polyspermy block, when the structure of the ZP is modified by enzymes of cortical granules. Martinova *et al.* (2008) have shown that after fertilization the ZP undergoes visible structural changes. Future studies must address if there is a different distribution of TFD between the inner and outer layer of ZP and if this distribution changes after fertilization and during oviduct passage. Furthermore, the protein/glycoresidue binding groove of TFD could be responsible for binding carbohydrates of other glycoproteins. For example, other trefoil factors interact specifically with mucins (Kindon *et al.*, 1995) and the mammalian oviduct secretes proteins which contain a mucin-like domain. These oviductins are suggested to interact with the ZP, producing a protective layer around the oocyte and early embryo (Malette *et al.*, 1995). Thus, TFD might act as binding partners for oviductins.

To summarize, the suggested sperm receptor function of the trefoil domain within pig ZP could not be proven. Since fertilization is a multi-step event, the investigation of this process must be performed also on multiple levels as demonstrated within this paper. Further research is necessary to identify the function of TFD within the ZP; a structural role during oviduct passage is suggested.

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