Article

# Role of proteasomal activity in the induction of acrosomal exocytosis in human spermatozoa

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## Abstract

Sperm-associated proteasomes have been suggested to play an important role during fertilization in animals. To delineate the role of these proteasomes during fertilization in humans, the present study reports proteasomal proteolytic activity both in non-capacitated and capacitated human spermatozoa, which is not altered in the presence of baculovirus-expressed recombinant human zona pellucida glycoprotein-3 (ZP3) and zona pellucida glycoprotein-4 (ZP4). However, inhibition of proteasomal proteolytic activity by clasto-lactacystin β-lactone (CLBL) and Z-Leu-Leu-Leu-CHO (MG132), which are specific inhibitors of the 20S proteasomal core proteases, led to a significant ($P < 0.05$) inhibition of induction of acrosome reaction mediated by both recombinant human ZP3 and ZP4. Both inhibitors, however, failed to inhibit the induction of acrosomal exocytosis mediated by pharmacological agonist, calcium ionophore (A23187). The binding of recombinant human ZP3 and ZP4, labelled with fluorescein isothiocyanate, to the capacitated spermatozoa was not affected in the presence of proteasomal inhibitors. These observations suggest a role of the sperm proteasome in the induction of ZP3- and ZP4-mediated acrosomal exocytosis upstream of calcium signalling in humans.

***Keywords:*** *acrosome reaction, capacitation, proteasome, recombinant human zona pellucida glycoprotein, spermatozoa*

## Introduction

Mammalian oocytes are surrounded by an acellular glycoproteinaceous matrix termed zona pellucida (ZP) that plays a pivotal role in species-specific sperm–egg recognition, binding, induction of the acrosome reaction (AR) in the ZP-bound spermatozoa, anti-polyspermy defence and protection of the embryo prior to implantation. Biochemical characterization of the ZP matrix from various mammalian species revealed that it is composed of three or four glycoproteins (Spargo and Hope, 2003; Conner *et al*., 2005). Recent studies revealed that human ZP comprises four glycoproteins designated as ZP1, ZP2, ZP3 and ZP4 (Lefievre *et al*., 2004; Conner *et al*., 2005; Ganguly *et al*., 2008), as compared with three glycoproteins in the mouse ZP (Harris *et al*., 1994). Several studies suggest that in the mouse, ZP3 serves as the putative primary sperm receptor and is also responsible for inducing acrosomal exocytosis in the capacitated spermatozoa (Bleil and Wassarman, 1980; Beebe *et al*., 1992). In the mouse model, ZP2 has been shown to serve as the secondary sperm receptor and maintains the binding of the acrosome-reacted spermatozoa to the ZP matrix (Bleil *et al*., 1988).

The critical appraisal of the role of human ZP3 and other ZP glycoproteins during fertilization has been hindered due to their paucity in a highly purified form from a native source, as large numbers of human oocytes are not available for research purposes due to ethical considerations. In order to circumvent this limitation, over the years, several groups have successfully expressed and purified recombinant human ZP glycoproteins in various heterologous expression systems. Recombinant human

ZP3 expressed in Chinese hamster ovary (CHO) cells, was used to demonstrate that incubation of capacitated human spermatozoa with CHO-expressed human ZP3 leads to induction of acrosomal exocytosis (van Duin *et al.*, 1994). Recently, baculovirus-expressed recombinant human ZP glycoproteins, has been used to demonstrate that both ZP3 and ZP4 bind to the anterior head of the capacitated spermatozoa, whereas ZP2 binds in the equatorial region of the acrosome-reacted spermatozoa (Chakravarty *et al.*, 2008). Additionally, treatment of capacitated human spermatozoa with baculovirus-expressed recombinant human ZP3 and ZP4 led to induction of acrosomal exocytosis in both dose- and time-dependent manners (Chakravarty *et al.*, 2005).

Several candidate ligands have been characterized on spermatozoa that are involved in binding to ZP glycoproteins as well as induction of sperm acrosomal exocytosis (Wassarman, 1999). Recently, the 26S proteasome complex, present on spermatozoa, has been shown to play an important role during fertilization in various mammalian species (Morales *et al.*, 2003; Sutovsky *et al.*, 2003, 2004; Pizarro *et al.*, 2004; Yi *et al.*, 2007a). In the porcine system, one or more of the ZP glycoproteins are ubiquitinated (Sutovsky *et al.*, 2004). While these proteasomes seem to be associated with the outer acrosomal membrane and acrosomal matrix, acrosomal exocytosis results in the exposure of proteasomes associated with the inner acrosomal membrane that could participate in the proteolysis of the ZP matrix during sperm–zona penetration (Sutovsky *et al.*, 2004). The location of proteasome in mature human spermatozoa was restricted to the sperm tail, connecting piece, acrosomal, and post-acrosomal regions (Wojcik *et al.*, 2000; Bialy *et al.*, 2001; Morales *et al.*, 2004). Use of proteasome-specific inhibitors led to a block in ZP-induced AR, suggesting a role for proteasomes in human fertilization (Morales *et al.*, 2003). However, it is not known which ZP protein triggers the proteasome-dependent step of AR.

In the present study, the activity of proteasomes has been assessed in capacitated and non-capacitated human spermatozoa. Employing baculovirus-expressed recombinant human ZP3 and ZP4, attempts have been made to further understand the role of sperm proteasome in ZP3- and ZP4-mediated AR using proteasome-specific inhibitors. Furthermore, the effect of the above proteasomal inhibitors on the binding of baculovirus-expressed recombinant human zona proteins *per se* to the capacitated human spermatozoa has been assessed.

# Materials and methods

## Recombinant human zona proteins

The cloning and expression of recombinant human ZP3 (amino acid residues 1–424) and ZP4 (amino acid residues 1–540) as polyhistidine-tagged fusion proteins in the baculovirus expression system has previously been reported (Chakravarty *et al.*, 2005). For large scale production of the recombinant proteins, a suspension culture of 50 × $10^6$ *Spodoptera frugiperda* (*Sf21*) insect cells growing in a Spinner bottle (Thermolyne, Barnstead International, Iowa, USA) were incubated with the recombinant virus at a multiplicity of infection (MOI) of 3 at 42 rotations per minute (rpm) on a biological stirrer (Thermolyne) at 27°C. After 96 h of incubation, cells were pelleted at 1000 *g* for 15 min, and purified for recombinant human ZP3 and ZP4 using Ni-NTA resin as described previously (Gahlay and Gupta, 2003). The purified recombinant human zona proteins in 20 mmol/l Tris (pH 7.4) were assessed for protein concentrations using bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as the standard. The purified recombinant baculovirus-expressed human ZP3 and ZP4 were conjugated at a molar ratio of 1:24 with fluorescein isothiocyanate (FITC) Isomer I (Pierce) as described previously (Chakravarty *et al.*, 2008). The molar fluorescein/recombinant protein (F/P) ratios for ZP3 and ZP4 were 1.60 and 1.06 respectively (Chakravarty *et al.*, 2008). The FITC conjugated recombinant human zona proteins were assessed for purity by resolving on a 0.1% sodium dodecyl sulphate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) under reducing conditions followed by Coomassie blue staining essentially as described previously (Chakravarty *et al.*, 2005).

## Characterization of sperm proteasomal–proteolytic activity before and after capacitation

All experiments that used human spermatozoa were carried out with informed consent and following clearance from the Institutional Bio-safety and Human Ethical Committee. The semen samples were donated by fertile, healthy, consenting donors and subjected to liquefaction at room temperature for 30 min. The motile spermatozoa were separated by a two-step Percoll density gradient (Suarez *et al.*, 1986) and processed for capacitation in Biggers–Whitten–Whittingham (BWW) medium (Biggers, 1971) supplemented with 2.6% BSA (cell culture grade; Sigma Chemical Co., St Louis, MO, USA) as described previously (Chakravarty *et al.*, 2005).

A fluorogenic proteasomal substrate Z-Leu-Leu-Glu-7-amino-4-methylcoumarin (Z-LLE-AMC; Boston Biochem, Cambridge, MA, USA), susceptible to peptidylglutamyl peptide-hydrolysing activity of the 20S proteasomal core, was used to assess spermatozoa proteasome activity before and after capacitation. Samples of 80 µl of either non-capacitated or capacitated spermatozoa (10 × $10^6$ sperm/ml) were incubated in Tris-buffered medium (113.1 mmol/l NaCl, 3 mmol/l KCl, 7.5 mmol/l $CaCl_2$, 11 mmol/l glucose and 5 mmol/l sodium pyruvate) with 100 µmol/l of Z-LLE-AMC in a final reaction volume of 100 µl. The reaction mixture was incubated at 37°C in a Fluostar Optima Spectrofluorometer (BMG Technologies, Offenburg, Germany) and the fluorescence monitored after optimized time intervals of 5 min each during a 60-min incubation period at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. In addition, the sperm proteasomal activity on Z-LLE-AMC was also assessed in the presence of recombinant human ZP3 and ZP4 (2 µg/reaction) and 100 µmol/l of proteasome-specific inhibitor Z-Leu-Leu-Leu-CHO (MG132; Biomol Research Labs., Plymouth Meeting, PA, USA).

## Evaluation of the binding of recombinant human zona proteins to human spermatozoa in presence of proteasome-specific inhibitors

The 5 × $10^6$ capacitated spermatozoa obtained as described above were incubated in BWW medium supplemented with 0.3% BSA in a total reaction volume of 50 µl with 2.5 µg of FITC

conjugated recombinant human ZP3 and ZP4 in the presence or absence of 100 µmol/l of clasto-lactacystin-beta-lactone (CLBL) or 100 µmol/l MG132 for an optimized duration of 15 min at 37°C with 5% $CO_2$ in humidified air, followed by incubation at 4°C for 30 min. Appropriate control reactions were also set up wherein dimethyl sulphoxide (DMSO) for CLBL and ethanol for MG132 replaced the respective inhibitor in the reaction mixture. Spermatozoa were pelleted at 500 *g* for 5 min, washed with 50 mmol/l phosphate buffered saline (PBS) pH 7.4 and fixed in 4% paraformaldehyde for 30 min at room temperature. The fixed spermatozoa were washed once with 50 mmol/l PBS pH 7.4 and 10 µl aliquots were spotted onto poly-L-lysine-coated slides (Sigma). The spots were air-dried and spermatozoa analysed for acrosome status by staining with 5 µg/ml *Pisum sativum* agglutinin (PSA) conjugated to tetramethylrhodamine isothiocyanate (TRITC–PSA, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Slides were then washed three times with PBS and observed for double staining (fluorescence) under a Nikon Eclipse 80i epifluorescence microscope (Nikon, Chiyodu-ku, Tokyo, Japan) using an oil immersion objective. Any spermatozoa that displayed complete loss of PSA staining in the acrosome or revealed staining at the equatorial region were classified as acrosome-reacted. Spermatozoa showing PSA staining in the acrosome were scored as acrosome-intact. In all the binding experiments, at least 200 spermatozoa were scored for each reaction and the experiment was repeated three times with semen samples from at least two different donors.

## Induction of acrosome reaction by recombinant human ZP3 and ZP4 in presence of proteasome-specific inhibitors

Capacitated human spermatozoa ($1 \times 10^6$ in BWW + 0.3% BSA) obtained as described above were incubated with 2.0 µg of the respective recombinant human zona proteins at 37°C with 5% $CO_2$ in humidified air for 60 min in the absence or presence of 100 µmol/l of either CLBL or MG132 in a total reaction volume of 100 µl. In addition, capacitated spermatozoa were also incubated with 10 µmol/l of calcium ionophore (A23187; Sigma) in the absence and presence of CLBL and MG132. Spontaneous AR was assessed by incubation of the capacitated spermatozoa in the absence of recombinant human zona proteins. Post-incubation, the spermatozoa were washed with 50 mmol/l PBS pH 7.4, fixed in chilled methanol for 5 min and 25 µl aliquots were spotted on slides in duplicate. The spots were air-dried, stained with TRITC–PSA and processed as described above for analysis of the sperm acrosomal status.

## Statistical analysis

In the experiments pertaining to the characterization of proteasomal-proteolytic activity during capacitation, the results were expressed as mean ± SEM of ratio of absorbance ($\lambda_{ex}$ 380 nm and $\lambda_{em}$ 460 nm) of substrate Z-LLE-AMC at 60/0 min of three different experiments using semen samples from at least two different donors. The statistical analysis was performed by comparing the means of the sperm plus substrate and substrate alone within one experimental group by using two-tailed paired *t*-test and among three different groups using one-way analysis of variance (ANOVA) followed by Newmans–Keuls multiple comparison test. The results pertaining to the binding of baculovirus-expressed recombinant human zona proteins to the capacitated spermatozoa were also presented as mean ± SEM of at least three independent experiments. The statistical analysis of the experiments pertaining to the induction of acrosome reaction was performed by comparing the means of the medium control (BWW + 0.3% BSA) and experimental sets, or within two experimental groups as described above. A *P*-value of $< 0.05$ was considered to be statistically significant.

# Results

## Recombinant human ZP glycoproteins

The baculovirus-expressed recombinant human zona proteins were purified by Ni-NTA affinity chromatography and conjugated to FITC as described elsewhere (Chakravarty *et al.*, 2008). On SDS-PAGE analysis, the FITC labelled baculovirus-expressed human ZP3 and ZP4 revealed bands of ~65 and ~75 kDa respectively (**Figure 1**).

## Characterization of proteasomal–proteolytic activities in the non-capacitated and capacitated human spermatozoa co-incubated with recombinant human ZP3 and ZP4

Incubation of 6 h capacitated human spermatozoa ($0.8 \times 10^6$) with a specific proteasomal substrate Z-LLE-AMC (peptidylglutamyl peptide-hydrolysing activity of the 20S proteasomal core) for 60 min, demonstrated a linear increase in the absorbance ($\lambda_{ex}$ 380 nm and $\lambda_{em}$ 460 nm) (**Figure 2**). The ratio (60/0 min) of the absorbance in the presence of proteasome substrate was 2.52 ± 0.15 as compared with 0.51 ± 0.06 with substrate alone (**Table 1**). This activity was efficiently inhibited by proteasomal inhibitor, MG132 (**Table 1**, **Figure 2**). Capacitation of spermatozoa for 16 h showed a slight increase in the proteasome proteolytic activity, which was not significantly different from 6 h capacitated spermatozoa (**Table 1**). Interestingly, non-capacitated spermatozoa also showed proteasome proteolytic activity that was not significantly different from the capacitated spermatozoa (**Table 1**). Inclusion of recombinant human ZP3 as well as ZP4 along with the spermatozoa did not significantly alter the proteasomal proteolytic activity (**Table 1**, **Figure 2**).

## Effect of proteasomal inhibitors on binding characteristics of recombinant human ZP glycoproteins to spermatozoa

In the presence of specific proteasomal inhibitors, the FITC-conjugated baculovirus-expressed recombinant human ZP glycoproteins were analysed for their ability to bind to capacitated human spermatozoa by direct binding assay. The results are presented in **Table 2** and the representative binding profiles of recombinant human ZP glycoproteins in the presence of CLBL are depicted in **Figure 3**. The acrosomal status of the spermatozoa showing the binding of the recombinant proteins was assessed simultaneously by using TRITC–PSA staining as described above. Binding studies with baculovirus-expressed recombinant

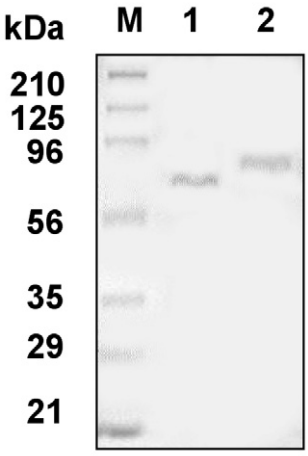


**Figure 1.** Analysis of purified recombinant human zona proteins conjugated to fluorescein isothiocyanate (FITC) by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The purified baculovirus-expressed recombinant human zona pellucida glycoprotein-3 (ZP3) and zona pellucida glycoprotein-4 (ZP4) conjugated to FITC were resolved by 0.1% SDS-10% PAGE under reducing conditions and analysed by Coomassie blue staining. Lanes are represented as M: molecular weight markers; lanes 1–2: FITC labelled recombinant human ZP3 and ZP4 respectively (5 $\mu$g/lane).

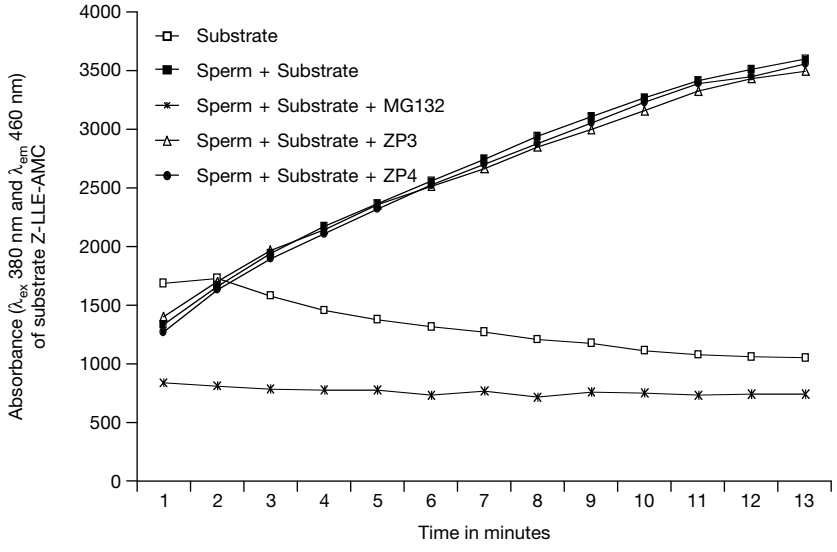


**Figure 2.** Measurement of proteasome–proteolytic activity in the capacitated human spermatozoa. Capacitated human spermatozoa ($0.8 \times 10^6$) were incubated at 37ºC with 100 $\mu$mol/l of fluorochrome 26S proteasome substrate Z-Leu-Leu-Glu-7-amino-4-methylcoumarin (Z-LLE-AMC) either in the absence or presence of 100 $\mu$mol/l of proteasome specific inhibitor Z-Leu-Leu-Leu-CHO (MG132) as described in Materials and methods. In addition, capacitated human spermatozoa were also incubated with Z-LLE-AMC substrate along with baculovirus-expressed recombinant human ZP3 and ZP4 (2.0 $\mu$g/reaction). The absorbance of the substrate ($\lambda_{ex}$ 380 nm and $\lambda_{em}$ 460 nm) was measured over a span of 60 min.

**Table 1.** Proteasomal proteolytic activity in non-capacitated and capacitated human spermatozoa in the presence and absence of baculovirus-expressed recombinant human zona pellucida glycoprotein-3 (ZP3) and ZP4.

| *Experimental condition* | *Ratio of absorbance at 60/0 min (mean ± SEM)*[a] | P-*value* |
|---|---|---|
| *Non-capacitated spermatozoa* | | |
| Substrate alone | 0.60 ± 0.02 | – |
| Sperm + substrate | 2.48 ± 0.12 | $P < 0.01$[e], NS[g] |
| Sperm + substrate + MG132[b] | 0.47 ± 0.05 | – |
| Sperm + substrate + ZP3[c] | 2.32 ± 0.09 | NS[f,g] |
| Sperm + substrate + ZP4[d] | 2.43 ± 0.07 | NS[f,g] |
| *6 h capacitated spermatozoa* | | |
| Substrate alone | 0.51 ± 0.06 | – |
| Sperm + substrate | 2.52 ± 0.15 | $P < 0.01$[e], NS[g] |
| Sperm + substrate + MG132[b] | 0.44 ± 0.04 | – |
| Sperm + substrate + ZP3[c] | 2.54 ± 0.06 | NS[f,g] |
| Sperm + substrate + ZP4[d] | 2.29 ± 0.24 | NS[f,g] |
| *16 h capacitated spermatozoa* | | |
| Substrate alone | 0.50 ± 0.06 | – |
| Sperm + substrate | 2.71 ± 0.05 | $P < 0.001$[e], NS[g] |
| Sperm + substrate + MG132[b] | 0.68 ± 0.12 | – |
| Sperm + substrate + ZP3[c] | 2.63 ± 0.07 | NS[f,g] |
| Sperm + substrate + ZP4[d] | 2.73 ± 0.05 | NS[f,g] |

[a]Proteasomal proteolytic activity is expressed as 60/0 min ratio of absorbance calculated by dividing proteasomal substrate Z-Leu-Leu-Glu-7-amino-4-methylcoumarin (Z-LLE-AMC) absorbance ($\lambda_{ex}$ 380 nm and $\lambda_{em}$ 460 nm) at 60 min post-incubating reaction mixture with substrate, by absorbance at 0 min. Each value represents mean ± SEM of three independent experiments with semen samples from at least two different donors.
[b]Capacitated/non-capacitated spermatozoa ($0.8 \times 10^6$) were incubated with 100 $\mu$mol/l substrate in presence of 100 $\mu$mol/l proteasome inhibitor Z-Leu-Leu-Leu-CHO (MG132) for 60 min at 37°C.
[c]Capacitated/non-capacitated spermatozoa ($0.8 \times 10^6$) were incubated with 100 $\mu$mol/l substrate in presence of recombinant human ZP3 (2.0 $\mu$g/100 $\mu$l).
[d]Capacitated/non-capacitated spermatozoa ($0.8 \times 10^6$) were incubated with 100 $\mu$mol/l substrate in presence of recombinant human ZP4 (2.0 $\mu$g/100 $\mu$l).
[e]Two-tailed paired *t*-test between substrate alone and sperm + substrate group.
[f]Two-tailed paired *t*-test between sperm + substrate and sperm + substrate + ZP3/ZP4 group.
[g]One-way analysis of variance followed by Newmans–Keuls multiple comparison test among non-capacitated, 6 and 16 h capacitated sperm groups.
NS = not statistically significant.

proteins in the presence of proteasomal inhibitors revealed that 19.50 ± 2.10% (mean ± SEM) and 18.00 ± 1.50% of capacitated spermatozoa exhibited binding of recombinant ZP3 to sperm acrosome in the presence of CLBL and MG132 respectively (**Table 2**). In the absence of proteasomal inhibitors, 19.08 ± 2.05% spermatozoa showed the binding of ZP3 to capacitated spermatozoa (**Table 2**). Two distinct binding patterns were observed in all the above groups: in the acrosomal cap and in the equatorial region (**Figure 3**). The relative distribution of such binding patterns is described in **Table 2**. A higher percentage of the spermatozoa exhibited binding of recombinant human ZP3 to the equatorial region as compared with the acrosomal cap (**Table 2**). The binding profiles of recombinant human ZP3 to the capacitated spermatozoa in the presence of proteasome-specific inhibitors were identical to the results obtained in the absence of either inhibitor.

Similar results were obtained for the evaluation of binding characteristics of capacitated spermatozoa with recombinant human ZP4 conjugated to FITC in the presence of CLBL and MG132 (**Table 2**). However, in contrast to ZP3, FITC-labelled ZP4 showed higher binding on the acrosomal cap as compared with the equatorial region of the capacitated spermatozoa (**Table 2**).

## Effect of proteasome-specific inhibitors on the induction of acrosome reaction by recombinant human ZP3 and ZP4

Since the binding of the recombinant human zona proteins to spermatozoa was not inhibited in the presence of proteasome-specific inhibitors, it was prudent to investigate whether the

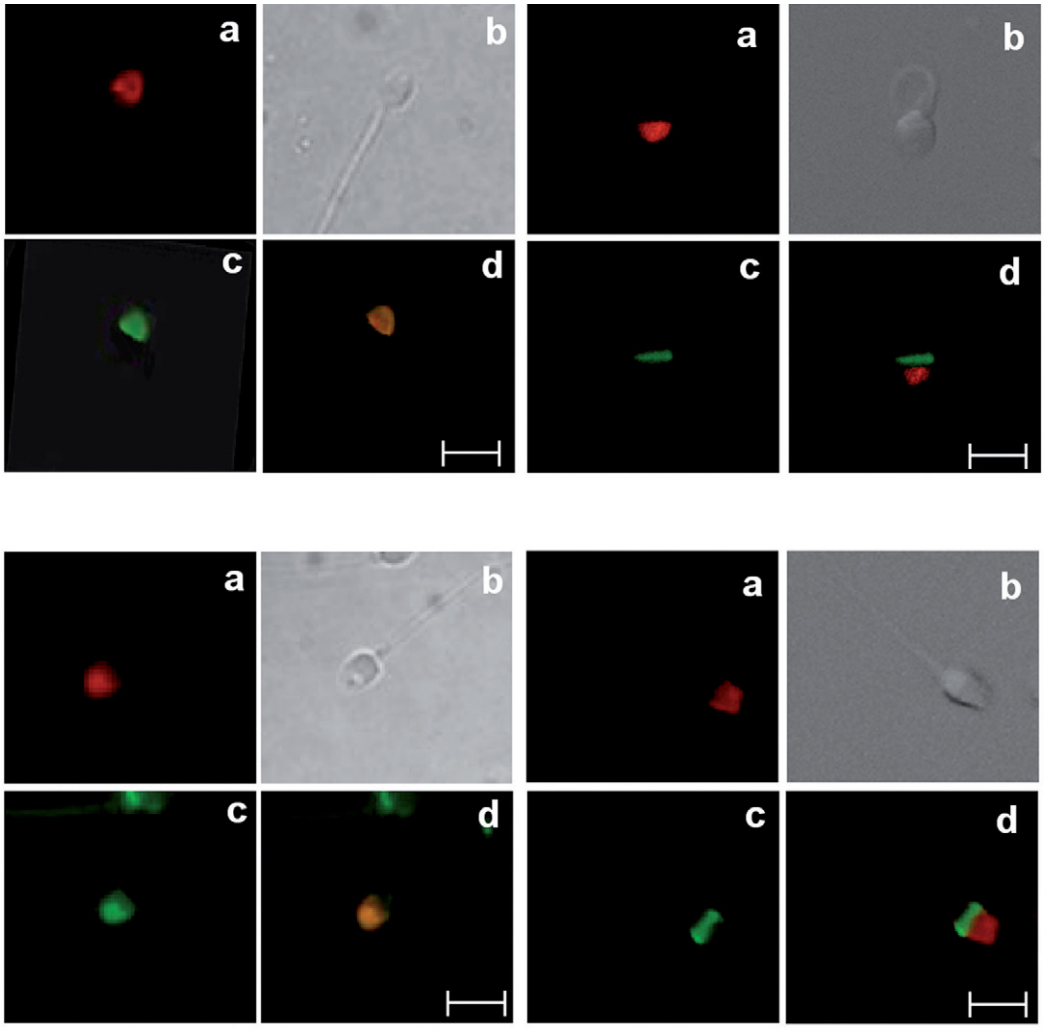


**Figure 3.** Binding profile of recombinant human zona pellucida glycoprotein-3 (ZP3) and ZP4 with human spermatozoa in the presence of proteasome-specific inhibitor. Capacitated spermatozoa ($5 \times 10^6$/50 $\mu$l) were incubated with fluorescein isothiocyanate (FITC) conjugated recombinant ZP3 (upper panel) or ZP4 (lower panel) in the presence of clasto-lactacystin β-lactone (CLBL) (100 $\mu$mol/l) and processed for analysis of their binding as described in Materials and methods. The acrosomal status was determined by labelling the spermatozoa with tetramethyl-rhodamine isothiocyanate conjugated *Pisum sativum* agglutinin (TRITC–PSA). Upper and lower left panels show the representative binding profiles of FITC-conjugated ZP3 and ZP4 respectively to the acrosomal cap whereas upper and lower right hand panels shows the representative binding profiles to the equatorial region. In each panel, the sub-panels are represented as (**a**) TRITC–PSA fluorescence; (**b**) phase contrast; (**c**) FITC–ZP3/ZP4 fluorescence; and (**d**) overlap of fluorescent frames. The scale bar represents 2.5 $\mu$m.

**Table 2.** Binding characteristics of baculovirus-expressed recombinant human zona pellucida glycoprotein-3 (ZP3) and ZP4 with capacitated human spermatozoa in the presence or absence of proteasomal inhibitors.

| *Treatment*[a] | *Percent binding to spermatozoa* | *Binding patterns Acrosomal cap (%)* | *Equatorial region (%)* |
|---|---|---|---|
| ZP3 | 19.08 ± 2.05 | 36.10 | 63.90 |
| ZP3 + CLBL | 19.50 ± 2.10 | 31.79 | 68.21 |
| ZP3 + MG132 | 18.00 ± 1.50 | 38.15 | 61.85 |
| ZP4 | 17.95 ± 2.37 | 87.90 | 12.10 |
| ZP4 + CLBL | 15.98 ± 3.22 | 85.65 | 14.35 |
| ZP4 + MG132 | 17.23 ± 1.04 | 77.60 | 22.00 |

[a]Capacitated spermatozoa were incubated with the fluorescein isothiocyanate labelled recombinant proteins at a concentration of 2.5 µg/50 µl in the absence/presence of 100 µmol/l of proteasome-specific inhibitors, clasto-lactacystin β-lactone (CLBL)/Z-Leu-Leu-Leu-CHO (MG132) and processed for analysis of binding, as described in Materials and methods.

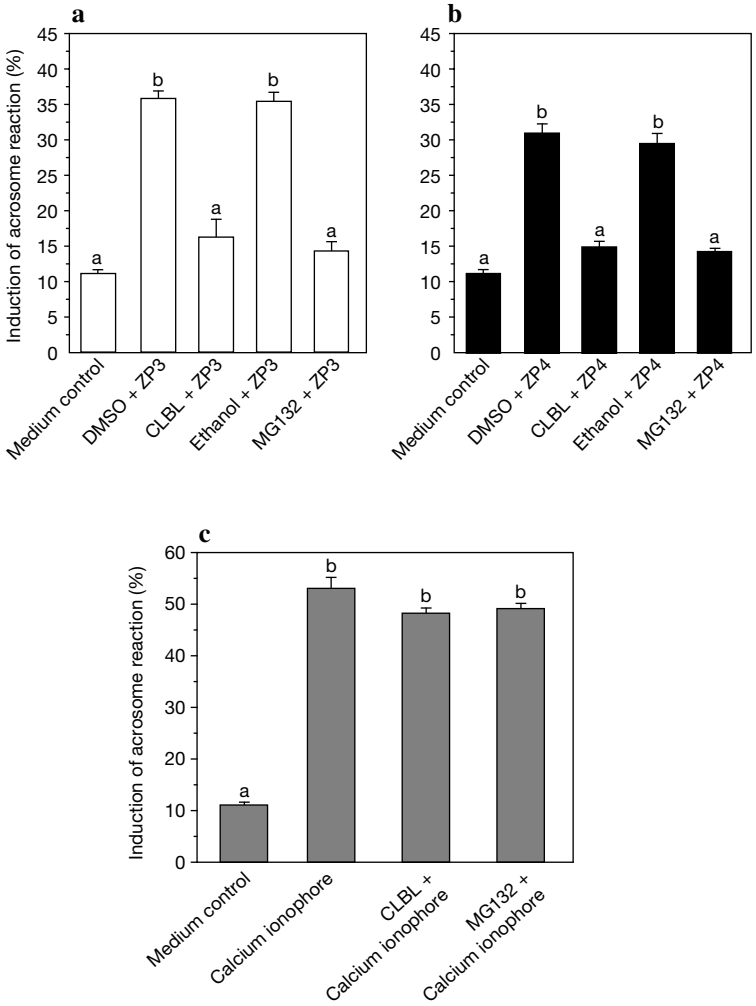


**Figure 4.** Analysis of the ability of baculovirus-expressed recombinant human ZP3 and ZP4 to induce acrosome reaction in the presence of proteasome-specific inhibitors in capacitated human spermatozoa. Capacitated spermatozoa ($1 \times 10^6$/100 $\mu$l) were incubated with (**a**) recombinant human ZP3 (2.5 $\mu$g/100 $\mu$l), (**b**) recombinant human ZP4 (2.5 $\mu$g/100 $\mu$l) and (**c**) calcium ionophore (10 $\mu$mol/l) in the presence of 100 $\mu$mol/l of the proteasome-specific inhibitors, clasto-lactacystin β-lactone (CLBL) and Z-Leu-Leu-Leu-CHO (MG132) independently, or their respective control solvents for 1 h and subsequently analysed for acrosomal status by tetramethyl–rhodamine isothiocyanate-conjugated *Pisum sativum* agglutinin (TRITC–PSA) staining as described in the Materials and methods section. Values are expressed as mean ± SEM of three different experiments using semen samples from at least two male donors. Bars within each panel that are labelled with different letters identify means that are statistically significantly different.

induction of acrosome reaction mediated by recombinant human zona proteins was affected by the presence of proteasome-specific inhibitors. These studies, performed as described above, revealed that presence of either of the proteasome-specific inhibitors individually at a concentration of 100 μmol/l, abrogated the induction of acrosomal exocytosis in capacitated human spermatozoa by baculovirus-expressed recombinant ZP3 as well as ZP4 in a statistically significant manner (**Figure 4a**,**b**). The calcium ionophore-induced acrosome reaction, however, remained unaffected in the presence of both inhibitors used independently (**Figure 4c**).

## Discussion

Spermiogenesis, a highly regulated process, involves a complex interplay of chromatin remodelling, cell-specific gene expression and downstream signalling cascade, resulting in transformation of spermatogonia to spermatozoa (Krausz and Sassone-Corsi, 2005). A spermatozoon with normal morphology is a prerequisite for successful fertilization. However, recent reports have suggested that chemical activation of the human oocyte after intracytoplasmic sperm injection (ICSI) in selected cases with a previous history of failed or low fertilization associated with spermatozoa abnormality, may improve the success rate of fertilization (Moaz *et al.*, 2006). It is a matter of discussion whether the fertilizing mammalian spermatozoa penetrate the egg coat solely by mechanical force, by enzymatic proteolytic activity associated with the sperm acrosome, or by a combination of the mechanical and enzymatic forces (Bedford, 1998; Olds-Clarke, 2003; Yi *et al.*, 2007a). Several studies have implicated sperm-borne proteasomes in the process of sperm penetration through the egg coat in various mammalian and non-mammalian species (reviewed by Sakai *et al.*, 2004). The 26S proteasome is a multi-subunit protease, targeting proteins post-translationally modified by ubiquitination (Glickman and Ciechanover, 2002). Current studies are investigating whether the sperm proteasomes participate in acrosomal exocytosis and/or in the digestion of ZP matrix during mammalian fertilization. During IVF in the porcine model, the inhibition of sperm proteasomal activity prevents sperm–ZP penetration without affecting sperm motility and sperm–ZP binding (Sutovsky *et al.*, 2003, 2004). Inversely, the inhibition of proteasome-associated deubiquitinating activity, believed to stimulate proteasomal proteolysis (Guterman and Glickman, 2004), increases the rate of polyspermic fertilization in the pig (Yi *et al.*, 2007b). The proteasomal requirement for penetration of the egg coat by spermatozoa appears to be evolutionarily conserved. The ascidian sperm receptor HrVC70, a homologue of mouse ZP3, is sequentially ubiquitinated and degraded by the sperm-borne proteasomes to facilitate sperm passage through the vitelline envelope (Sawada *et al.*, 2002). In humans and rodents, acrosomal exocytosis seems to depend upon proteasomal proteolytic activity within the sperm acrosome (Morales *et al.*, 2003; Pizarro *et al.*, 2004; Pasten *et al.*, 2005).

Sperm-associated proteasomes have been documented in humans and various animal species by immunocytochemical, biochemical and proteomic approaches (Bialy *et al.*, 2001; Pizarro *et al.*, 2004; Sutovsky *et al.*, 2004). It is important to note that sperm proteasomes are exposed on the acrosomal surface even before acrosomal exocytosis (Pasten *et al.*, 2005), and therefore could interact directly with zona proteins during fertilization. The peptidyl-glutamyl peptidase activity of the 20S proteasomal core, observed in the capacitated human spermatozoa in the present study, is consistent with a previous report (Morales *et al.*, 2003). It is being examined whether the stimulation of sperm proteasomal activity occurs during capacitation. Similarly, it is not clear which acrosomal proteins could be degraded by the acrosomal proteasomes. Various groups have shown immunoreactivity of spermatozoa with antibodies against ubiquitin, proteasomes, ubiquitin-conjugating enzymes and deubiquitinating enzymes (Sutovsky, 2003). The peripheral acrosomal membrane proteins such as spermadhesins are among possible candidates for ubiquitinated acrosomal surface protein. This notion is supported by the recent finding that in boar sperm extracts spermadhesin AQN1 co-immunoprecipitates with proteasome-associated deubiquitinating enzyme UCHL3 (Yi *et al.*, 2007b). In addition to the participation of sperm proteasomes in AR and sperm–ZP penetration, they could be involved in the induction of capacitation, during which at least one spermadhesin, the AQN1, appears to be rapidly degraded (Ekhlasi-Hundrieser *et al.*, 2005). However, the present study show that the sperm-associated proteasomal–proteolytic activity remains steady during capacitation.

In the present study, induction of acrosomal exocytosis in the capacitated human spermatozoa by baculovirus-expressed recombinant human ZP3 and ZP4 (Chakravarty *et al.*, 2005) was efficiently inhibited by specific small molecule proteasomal inhibitors MG132 and CLBL, but not by control vehicle solutions. Inhibition in the induction of AR mediated by solubilized isolated human ZP or progesterone by proteasome inhibitors has also been shown previously (Morales *et al.*, 2003). Although this work agrees with published studies, the utilization of isolated zonae by Morales *et al.* (2003) introduced possible sources of variability. The experimental system relies on well-defined, recombinant zona proteins to provide definitive evidence for the requirement of proteasomal activity for ZP3/ZP4-induced AR. It is likely that the baculovirus-expressed recombinant ZP3 and ZP4 may not mimic glycosylation of naturally occurring ZP3 and ZP4 produced by the human oocyte, which warrants further investigation into the extent to which proteasomal activity could be modulated by highly pure, properly glycosylated ZP proteins.

The induction of AR with calcium ionophore was not affected by proteasomal inhibitors, suggesting that the proteasome-dependent step of acrosomal exocytosis is completed upstream of calcium influx into sperm acrosome, shown to be stimulated by sperm–ZP binding (reviewed by Darszon *et al.*, 2002). Sperm-associated proteasomal activity was not altered significantly by recombinant human ZP3 and ZP4, suggesting that it may not be stimulated by binding to ZP and it may remain steady after acrosomal exocytosis, possibly due to the association of the proteasomes with the inner acrosomal membrane that is not removed by AR. Accordingly, treatment with proteasomal inhibitors had no effect on recombinant human ZP3 and ZP4 binding to sperm acrosome, which is consistent with the lack of inhibitory effect on sperm-ZP binding during porcine IVF (Sutovsky *et al.*, 2004). However, both CLBL and MG132 applied at 100 μmol/l concentration inhibited acrosomal exocytosis in capacitated human spermatozoa in the present study. Inhibition of acrosomal exocytosis by MG132 was not observed in the porcine studies, whereas the passage of the spermatozoa was prevented with almost 100% efficiency by 10

μmol/l MG132 (i.e. a 10-fold lower MG132 concentration than used in the present study) or by the specific antibodies against the 20S proteasomal core subunits (Sutovsky *et al*., 2004). These comparisons suggest that the acrosomal exocytosis step of fertilization may be relatively less sensitive to proteasomal interference than that of the sperm–ZP penetration step.

Altogether, the data provide direct evidence for the requirement of proteasomal proteolysis during ZP3- and ZP4-induced acrosomal exocytosis in humans. The study identifies a ZP3- and ZP4-dependent early step in acrosomal exocytosis and open up an opportunity for targeting the sperm-borne ubiquitin–proteasome pathway to elicit a contraceptive effect.

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