

# Effects of Sperm Extract and its Molecular Weight Fractions on Oocyte Activation in Miniature Pig Spermatozoa

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**Abstract:** This study was conducted to compare the quantity of total protein in miniature pig sperm extract (SE) extracted by two methods [sonication and a method using Mammalian Protein Extraction Reagent (M-PER)]. Furthermore, the effects of injection with SE extracted by M-PER and fractionated SE on porcine oocyte activation were examined. The total amount of protein extracted from miniature pig spermatozoa ( $1 \times 10^9$  sperm) by M-PER was significantly ( $P < 0.05$ ) larger than that extracted by sonication. When the porcine oocyte activation rate after injection with SE extracted by M-PER at different protein concentrations was examined, the highest activation (31.4%) was induced by injection with 0.08 mg/ml SE, and the value was significantly ( $P < 0.05$ ) higher than the control (buffer injection). When the effect of injection with fractionated SE (>100 kDa and <100 kDa) was examined, the activation rates of the control (non-fraction), >100 kDa, and <100 kDa groups were 63.6, 48.7, and 46.4%, respectively. Significant differences ( $P < 0.05$ ) were observed between the control and <100 kDa groups. These results indicate that SE extracted by M-PER from miniature pig sperm is effective for porcine oocyte activation. Oocyte activation could be induced efficiently by injection with SE using M-PER. Both <100 kDa SE and >100 kDa SE contain effective materials for porcine oocyte activation.

**Key words:** Fractionated sperm extracts, Mammalian protein extraction reagent, Miniature pig spermatozoa, Oocyte activation, Porcine oocyte

## Introduction

It is well known that spermatozoa release cytosolic substances, called sperm factor (SF), into oocytes at fertilization and that SF induces  $[Ca^{2+}]_i$  oscillations [1–3]. Fissore *et al.* [4] reported that injection of sperm extracts from boar spermatozoa induced high rates of activation and cleavage in mouse and bovine oocytes. Injection of sperm extracts into mouse and bovine oocytes triggered  $[Ca^{2+}]_i$  rises that resemble spontaneous oscillation. Kimura *et al.* [5] designated sperm extracts as a sperm-borne oocyte-activating factor.

In our previous experiments [6], we investigated whether injection of sperm extracts (SE) extracted from miniature pig spermatozoa by sonication could induce porcine and bovine oocyte activation. The results showed that SE injection from miniature pig spermatozoa induced comparatively high rates of porcine and bovine oocyte activation. Furthermore, oocytes injected with SE exhibited cortical granule exocytosis as judged by FITC-PNA staining. From these results, we concluded that SE injection from miniature pig spermatozoa extracted by sonication into an oocyte was an effective method of activation.

There are various other methods for extraction of SF or SE from spermatozoa aside from sonication, such as freeze-thawing. Freezing was performed by directly plunging spermatozoa suspended in medium without a cryoprotectant into liquid nitrogen at  $-196^\circ\text{C}$ . They were then thawed by incubation at room temperature for 1 h [7]. As another method, Machaty *et al.* [8] recommended a method using Mammalian Protein Extraction Reagent (M-PER) for sperm treatment. They showed that a final concentration of 50–100 mg/ml of protein with a molecular weight greater than 100 kDa

Received: April 7, 2006

Accepted: June 6, 2006

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was obtained when the final sperm suspension (number of cells:  $3\text{--}10 \times 10^8$  /ml) was treated using M-PER.

If a large quantity of protein with high ability for oocyte activation was extracted from spermatozoa, it might improve the rate of oocyte activation; therefore, it is necessary to discover an effective extraction method. Purification and identification of SF and SE is also required for improvement of the oocyte activation rate.

The aims of this study were to compare the quality and quantity of SE extracted by two methods (sonication and M-PER) for porcine oocyte activation and to examine the effects of SE purified with a 2-D Clean Up Kit and fractionated SE on porcine oocyte activation.

## Materials and Methods

### *Sperm preparation*

Whole semen from Gottingen miniature pigs (2–3 years old) was collected once weekly by a glove hand technique and filtered through cotton gauze to remove gel particles. The semen was then centrifuged at  $400 \times g$  for 10 min to remove sediment. The supernatant was transferred into a new tube and centrifuged at  $700 \times g$  for 5 min. The sperm pellets were suspended and washed twice in TALP-Hepes (TL-Hepes) medium [9] at  $700 \times g$  for 5 min. The washed spermatozoa were resuspended in cell lysis buffer (75 mM KCl, 20 mM Hepes, 1 mM EDTA, 10 mM glycerophosphate, 1 mM DTT, 200  $\mu$ M PMSF, 10  $\mu$ g/ml pepstatin, 10  $\mu$ g/ml leupeptin; pH 7.0), and then the sperm concentration was adjusted to  $1 \times 10^9$ /ml.

### *Extraction of SE by sonication and its adjustment*

The extraction and adjustment methods of SE were adapted from the modified protocol of Wu *et al.* [10]. In brief, a sperm suspension was sonicated (UD-200, TOMY, Tokyo, Japan) for 35 min at 4°C in a dry ice alcohol mixture. The lysate was centrifuged twice at  $10,000 \times g$  and was further ultracentrifuged at  $100,000 \times g$  (TL-100, Beckman Coulter Fullerton, CA, USA) for 1 h at 4°C. The clear supernatant was filtered through a 3,000 molecular weight cut-off membrane (Microcon YM-3; Millipore Corporation, Bedford, MA, USA). The substances on the ultrafiltration membranes were washed twice with washing buffer solution consisting of 75 mM KCl and 20 mM Hepes (pH 7.0), and then the protein concentration of the diluted substances was adjusted to 1.0 mg/ml with the same buffer (see the following procedure). The adjusted substances were stored at  $-80^\circ\text{C}$  until use.

### *Adjustment of the protein concentration of SE*

BSA solutions with various concentrations (0.3, 0.6, 0.9, 1.2, and 1.5 mg/ml) and SE were treated using a DC Protein Assay Kit (Bio-Rad, San Francisco, CA, USA) according to the manufacturer's protocol. The absorbance of these solutions was measured using a spectrophotometer (V-520, SR type, JASCO Corporation, Tokyo, Japan). After making a standard curve for the BSA solutions, the protein concentration of the SE was determined based on the curve.

### *Extraction of SE by M-PER and its adjustment*

SE was extracted by M-PER (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. In brief, the sperm suspension was centrifuged at  $2,500 \times g$  for 10 min. A sperm pellet was obtained by discarding the supernatant, and M-PER was added to the pellet. At least 1 ml M-PER was used for each 100 mg of wet cell pellet. The lysate was shaken gently for 10 min by Mini Disk Rotor (BC-710; BIO CRAFT, Tokyo, Japan), and then it was centrifuged at  $14,000 \times g$  for 15 min to remove cell debris. The supernatant was transferred to a new tube for further treatment, which was the same as for the lysate obtained by sonication.

### *Treatment of the cytosolic fraction with a 2-D Clean Up Kit*

The aim of treatment with a 2-D Clean Up Kit (Amersham Biosciences Corp., San Francisco, CA, USA) was to remove detergents, salt, lipids, phenols, and nucleic acids from SE, according to the manufacturer's protocol. In brief, 300  $\mu$ l precipitant was added to 100  $\mu$ l SE and left on ice for 15 min. It was then centrifuged for 5 min at  $12,000 \times g$  to remove the supernatant, 25  $\mu$ l of distilled water was added to the pellet, and the mixture was vortexed for dispersal. After the addition of 1 ml chilled wash buffer and 5  $\mu$ l wash additive, the precipitate was collected by centrifugation for 5 min at  $15,000 \times g$ , resuspended with washing buffer, and washed twice. The sample was dissolved in injection buffer. Finally, the protein concentration of the sample was adjusted (see the above-mentioned procedure) to 6.0 mg/ml and stored at  $-80^\circ\text{C}$  until use.

### *Fractionation (more than 100 kDa or less than 100 kDa) of SE*

SE was extracted by sonication or M-PER, treated with a 2-D Clean Up Kit, and filtered through a 100,000 molecular weight cut-off membrane (Microcon YM-100; Millipore Corporation, Bedford, MA, USA). The substances on the membrane were washed twice with

washing buffer solution. Solutions containing these substances were defined as >100 kDa SE. On the other hand, substances passing through the membrane were defined as <100 kDa SE. The protein concentrations of both solutions were then adjusted to 1.0 mg/ml with the same buffer (see the above-mentioned procedure). The adjusted substances were stored at  $-80^{\circ}\text{C}$  until use.

#### *Porcine oocyte maturation*

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported within 2 h to the laboratory in 0.9% (w/v) saline solution containing 100 mg/ml kanamycin sulfate (Meiji Seika Kaisha, Ltd., Tokyo, Japan) at  $30^{\circ}\text{C}$ . The follicular contents were recovered by cutting them from visible small antral follicles (about 2–5 mm in diameter) with a razor and by scraping the inner surfaces of the follicle walls with a disposable surgical blade. Cumulus-oocyte complexes (COCs) with uniform ooplasm and a compact cumulus cell mass were placed in phosphate-buffered saline (PBS) containing 0.01% (w/v) polyvinylpyrrolidone (PVP) (Sigma, St. Louis, MO, USA). They were then washed twice with maturation medium (BSA-free NCSU37) [11] supplemented with 0.6 mM cysteine (Sigma), 10% FCS (Gibco BRL, Grand Island, NY, USA), 0.6  $\mu\text{g}/\text{ml}$  porcine FSH (Sigma), and 1.3  $\mu\text{g}/\text{ml}$  equine LH (Sigma). Twenty COCs were cultured for 48 h in a NUNC 48-well multidish (Nunc, Roskilde, Denmark) containing 300  $\mu\text{l}$  of maturation medium.

#### *Microinjection of SE into oocytes*

After cultivation of porcine COCs, cumulus cells were removed by pipetting. Matured oocytes with a first polar body were selected for SE injection. A Cell-Injector (Microinjector, CIJ-1, Shimadzu Corporation, Kyoto, Japan) and micromanipulator (Leitz, Germany) equipped with a Piezo-Impact Driving Unit (PMAS-CT110, Primetech, Tsuchiura, Ibaraki, Japan) were used for SE injection. The injection procedure was based on the protocol of Kimura and Yanagimachi [12]. The amount of injection was 5  $\mu\text{l}$ . Injection was performed on a heating stage at  $30^{\circ}\text{C}$ . The injected oocytes were transferred immediately into culture medium (TCM-199 containing 10% FCS) and incubated for 48 h at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  in air. Both nuclear formation and cleavage were evaluated after cultivation for 48 h.

#### *Evaluation of nuclear status*

After culture for 48 h, the oocytes were mounted on slides, compressed with cover slips, and fixed with

ethanol: acetic acid (3:1) for more than 48 h at room temperature. The fixed oocytes were stained with 1% lacmoid in 45% acetic acid. Nuclear status was examined using phase-contrast microscopy ( $\times 400$ ).

#### *Experimental design*

In experiment 1, SE was extracted by sonication or M-PER. The amount extracted by both methods was compared. In experiment 2, the effect of injection of SE extracted by M-PER on porcine oocyte activation was examined. Various concentrations (0.03, 0.08, 0.3, 0.8, and 4.0 mg/ml) of SE were injected into matured oocytes with a first polar body. In the control group, buffer solution consisting of 75 mM KCl and 20 mM HEPES was injected into matured oocytes. The rates of pronuclear formation and cleavage were examined after culture for 48 h. In experiment 3, the porcine oocyte activation rates after injection of non-treated SE (control) and SE treated with a 2-D Clean Up Kit (treatment) were compared. In experiment 4, SE purified with a 2-D Clean Up Kit was further fractionated (>100 kDa or <100 kDa). The fractionated and non-fractionated SE (control) were injected into porcine oocytes, and then activation was examined.

#### *Statistical analysis*

The data for activated oocytes was analyzed by  $\chi^2$  test. The other data was analyzed using one-way analysis of variance (ANOVA). All percentage data were subjected to arcsine transformation before statistical analysis. The data were compared using the Tukey-Kramer honestly significant difference test. All experiments were repeated at least three times. Statistical comparisons were performed using the JMP IN software program (SAS Institute Inc., Cary, NC, USA). Differences were considered significant at  $P < 0.05$ .

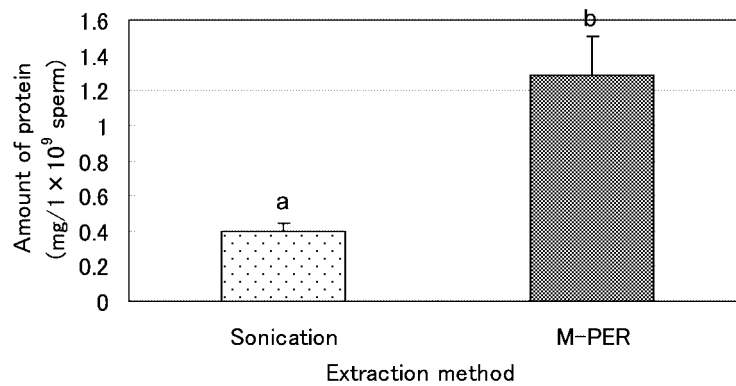
## **Results**

#### *Experiment 1*

Fig. 1 shows the total amount of protein extracted from miniature pig spermatozoa ( $1 \times 10^9$  sperm/ml) by sonication or M-PER. The latter (extraction by M-PER) was significantly ( $P < 0.05$ ) larger than the former (extraction by sonication).

#### *Experiment 2*

Table 1 shows the rates of pronucleus formation, cleavage, and activation after SE injection at different protein concentrations. The SE was extracted by M-



**Fig. 1.** Total amount of protein extracted from miniature pig spermatozoa ( $1 \times 10^9$  sperm) by sonication or M-PER. Values are the mean  $\pm$  SEM of three replications. <sup>a, b</sup>Different letters indicate significant differences ( $P < 0.05$ ).

**Table 1.** Porcine oocyte activation after injection of sperm extracts at different concentrations

Concentration of SE	No. of oocytes	No. of oocytes with pronucleus (%)	No. of cleaved oocytes (%)		No. of activated oocytes (Activation rate, %)
			$\geq 2$ cells	$\geq 8$ cells	
Buffer (control)	28	1 (3.6)	1 (3.6) <sup>a</sup>	0 (0.0)	2 (7.1) <sup>a</sup>
0.03 mg/ml	33	2 (6.1)	5 (15.2) <sup>b</sup>	2 (6.0)	9 (27.3)
0.08 mg/ml	35	3 (8.6)	6 (17.1) <sup>b</sup>	2 (5.7)	11 (31.4) <sup>b</sup>
0.3 mg/ml	31	1 (3.2)	3 (9.7)	1 (3.2)	5 (16.1)
0.8 mg/ml	38	4 (10.5)	3 (7.9)	2 (5.3)	9 (23.7)
4.0 mg/ml	34	1 (2.9)	4 (11.8)	1 (2.9)	6 (17.6)

Values within the same column with different letters (a, b) differ significantly ( $P < 0.05$ ).

PER.

The highest activation (31.4%) was induced by injection of 0.08 mg/ml SE, and the value was significantly ( $P < 0.05$ ) larger than for the control.

#### Experiment 3

The percentage of oocyte activation was significantly improved by purification using a 2-D Clean Up Kit (Fig. 2).

#### Experiment 4

As shown in Fig. 3, the activation rates of the control,  $>100$  kDa and,  $<100$  kDa groups were 63.4, 48.7, and 46.4%, respectively. Significant differences ( $P < 0.05$ ) were observed between the control and  $<100$  kDa groups.

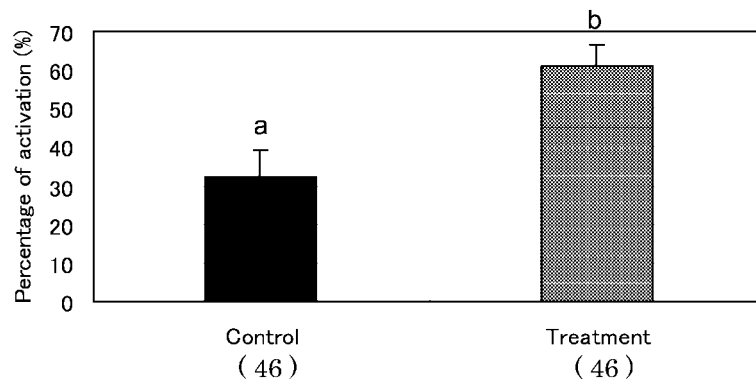
## Discussion

It has been clarified that sperm factor (SF) is a protein that exists in sperm extract (SE). SE injection can elicit

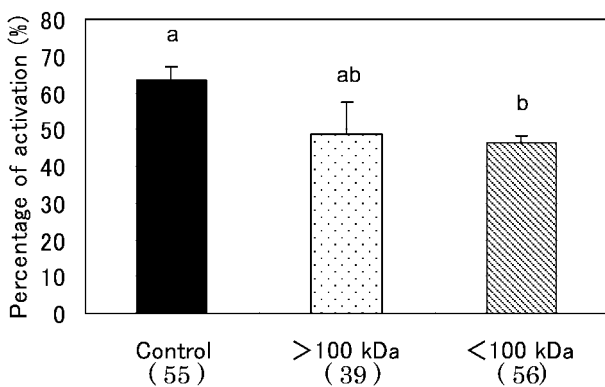
oocyte activation effectively [3, 4, 6, 10]. Extraction of significant effective protein from sperm is essential for oocyte activation.

In Experiment 1, SE extracted by sonication and M-PER were compared. The amount of protein extracted by M-PER was much larger than that extracted by sonication. The reason for this is unclear; however, there might have been a small amount of degeneration of SE extracted by M-PER because it was extracted under mild conditions, such as at room temperature.

The oocyte activation rate obtained from the results of Experiment 2 was 31.4% when oocytes were injected with 0.08 mg/ml SE. Our previous study [6] showed that injection of 0.08 mg/ml SE extracted by sonication induced oocyte activation at a rate of 20.4%. Furthermore, it showed that higher concentrations (0.3–0.8 mg/ml) of SE were required to reach an oocyte activation rate of 30%. Based on the present (Experiments 1 and 2) and previous results, we believed that M-PER might be superior to sonication for extraction of SF. As another advantage, we also noted



**Fig. 2.** Porcine oocyte activation after injection of non-treated SE (control) and SE treated with a 2-D Clean Up Kit (treatment). Values are the mean  $\pm$  SEM of three replications. The numbers of oocytes are indicated in parentheses. <sup>a, b</sup>Different letters indicate significant differences ( $P < 0.05$ ).



**Fig. 3.** Porcine oocyte activation after injection of non-treated SE (control) and fractionated SE (>100 kDa and <100 kDa). Values are the mean  $\pm$  SEM of three replications. The numbers of oocytes are indicated in parentheses. <sup>a, b</sup>Different letters indicate significant differences ( $P < 0.05$ ).

PLC $\zeta$  is expressed specifically in mouse spermatozoa and triggers  $[Ca^{2+}]_i$  oscillations in mouse oocytes [13]. As another candidate for SF, Sette *et al.* indicated that Tr-kit, a 23 kDa protein, is a truncated product of the c-kit gene and that it causes meiotic resumption through activation of phospholipase C $\gamma$ 1 (PLC $\gamma$ 1) and  $[Ca^{2+}]_i$  oscillations in mouse oocytes [14]. From these reports, it seems that SF has a low molecular weight and that low molecular materials are more effective than high molecular ones in SE for oocyte activation [13, 14]. Therefore, the effect of fractionation (>100 kDa or <100 kDa) of SE was investigated in porcine oocyte activation (Experiment 4). Contrary to our prediction, oocyte activation was successful for both >100 kDa SE and <100 kDa SE. This means that both >100 kDa SE and <100 kDa SE contain effective materials for oocyte activation. Machaty *et al.* [8] showed that >100 kDa SE extracted from boar sperm could induce porcine oocyte activation (activation rate of 51.7%), which is in agreement with the present results. Taking previous reports together with our results, it seems that SF, in addition to PLC $\zeta$  and Tr-kit, is present in SE extracted from the sperm of miniature pigs, and that the molecular weight of the SF is >100 kDa. It is unclear whether SF consists of one component or many components. Further work is required to clarify the component of SF.

In summary, extraction of sperm protein from miniature pig sperm using M-PER was effective. Oocyte activation could be induced efficiently by injection with SE extracted by M-PER. Both <100 kDa SE and >100 kDa SE contain some effective materials for porcine oocyte activation.

that the M-PER procedure was simpler than sonication. When SE was purified using a 2-D Clean Up Kit, the percentage of oocyte activation was significantly ( $P < 0.05$ ) improved (Experiment 3), indicating that purification with a 2-D Clean Up Kit is effective for improvement of the rate of oocyte activation. Although some studies have attempted to elucidate the molecular identity of SF protein, it still remains unknown. PLC $\zeta$  (zeta) is a candidate for SF. The molecular weight of PLC $\zeta$  is approximately 74 kDa.

### Acknowledgements

The authors thank the staff of the Meat Inspection Office of Hiroshima City for supplying the porcine ovaries.

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