Korean J. Animal Reprod. 26(4) : 369~375 (2002)

A Sperm Factor Inducing Second Polar Body Formation in Mouse Secondary Oocyte

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ABSTRACT

A sperm factor(s) for oocyte activation during fertilization has not been clearly identified. In this study to elucidate an oocyte activation factor(s), mouse sperm were sonicated and ultra-filtered with a 30 kilo-daltons (KD) cutoff membrane and the ultra-filtrate was then sequentially fractionated over Superose 12 column and Superdex column, The recovered fractions were micro-injected into M Ⅱ mouse oocytes and second polar body formation (PBF) was examined. Superose fraction RV2.10 prepared from sperm extract significantly increased PBF. Of Superdex fractions re-separated from Superose fraction RV2.10, fraction RV2.12 also had the strongest PBF activity. By analyzing with micro-reverse phase column (*u*RPC), the Superdex fraction RV2.12 appeared to be glutamic acid. In microinjection test, glutamic acid significantly increased PBF. This study suggests that glutamic acid should be a type of sperm factor for second polar body formation related to oocyte activation.

(Key words: Oocyte activation, Second polar body, Sperm factor, Glutamic acid)

Ⅰ. INTRODUCTION

Oocyte activation does not require only the fusion of the sperm-oocyte membrane (Maleszewski et al., 1995), but rather the release of a sperm factor (Tesarik et al., 1994). A factor for oocyte activation is contained in spermatozoa (Meng and Wolf. 1997). The sperm oocyte activation factor (SOAF) appears during transformation of the round spermatid into the spermatozoon (Kimura et al., 1998). It is primarily localized in the sperm head (Meng and Wolf. 1997) and might be of sperm head submembrane compartments (Perry et al., 1999). A SOAF released into the cytoplasm of the oocyte at the time of sperm-oocyte fusion (Stice and Robl, 1990) or during the sperm nuclear swelling phase (Dozortsev et al., 1997).

Many studies to elucidate the chemical properties of a SOAF show various results. A SOAF is reported to be possibly a heat sensitive and species-specific component (Dozortsev et al., 1995), or a high molecular weight protein (Swann, 1990). Wu et al. (1998) suggested that an oocyte activation factor of porcine sperm extracts were not glucosamine-6 -phosphate deaminase/oscillin but rather a compo-

This work was supported by a year 2000 grant of Kyungpook National University.

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nent with isoelectric point of 6.5～7.0 and relative molecular weight ranging from 29 to 68 KD. Stricker (1997) reported that more than 10 KD fractions of non-boiled sperm extracts retained full activity for oocyte activation, but not less than 10 KD fractions. Nonetheless, Osawa et al. (1994) suggested that an oocyte activation factor in the sperm extract survived heating at 100℃ for 10 minutes, pronase treatment, and that its molecular weight was less than 1,300 daltons. In a previous study, we reported that a SOAF was small molecule less than 300 daltons (Kim et al., 2000). Perry et al. (2000) suggested a model in which mammalian oocyte activation involves proteolytic processing of sperm head submembrane compartments.

As shown above, the characteristics of SOAF have been controversial to date. In this study to elucidate a SOAF, the sperm components were protected by extracting at 4℃ using the solution containing serine proteinase inhibitors and EDTA, but Ca^{2+} and Mg^{2+}, and finely separated using Smart system. The separated sperm components were micro-injected into MII stage oocytes and the second polar body formation (PBF) was examined as an index of oocyte activation.

Ⅱ. MATERIALS AND METHODS

1. Sperm Extract Preparation

Caudal epididymis was obtained from 15 to 16 weeks old ICR male mice that were proven as fertile. They were washed thrice with M_2 (Sigma Chemical Co., St. Louis, Mo. USA) and torn out with a needle to expose the sperm. The sperm were recovered and incubated at 37℃ for two hours, then centrifuged twice at 250×g for 10 minutes to wash the sperm in M_2.

The washed sperm were resuspended in sperm extraction solution (SES; 120mM KCl, 20mM HEPES, 2.5mM EDTA-2Na, 1mM benzamidin, 1mM phenylmethylsulfonyl fluoride, pH 7.0) and centrifuged at 250× g for 10 minutes. The sperm pellet was diluted with SES to recover 1×10^6 sperm. The sperm suspension was sonicated for two minutes at 4℃ using the UtrasonicLiquid Process VC502 (Sonic & Material Inc, U.S.A.). The sperm lysate was centrifuged at 10,000 × g for 30 minutes at 4℃ to recover a clear supernatant. The supernatant was centrifuged at 10,000×g for 30 minutes at 4℃ using Ultrafree-DEAE$_{MC}$ Filter Unit (M1661; Sigma Chemical Co., St. Louis, Mo. USA) and the ultra-filtrate containing components less than 30 kilo-daltons (KD) was recovered.

2. Separation of Sperm Components

The ultra-filtrate was loaded on a Superose 12 PC 3.2/30 (Pharmacia Biotech AB., Uppsala, Sweden) connected to the Smart system (Pharmacia Biotech AB., Uppsala, Sweden). Components of the ultra-filtrate were eluted with buffer A (120mM KCl, 20 mM HEPES, pH 7.0) at a flow rate of 40 μl/min and detected by UV monitor at OD_{280nm}. Of fractions automatically collected, half of each fraction was used for 2nd polar body formation (PBF) activity test and the other half was concentrated with N_2 for further re-separation as below.

The Superose fractions were loaded on Superdex Peptide PC 3.2/30 (Pharmacia Biotech AB., Uppsala, Sweden). The components of Superose fractions were eluted with buffer A at a flow rate of 100 μl/min and were detected by UV monitor at OD_{280nm}. Of each fraction, half was tested for their PBF activities and the other half was dried in a vacuum before re-separating with uRPC C2/C18 PC3.2/3(Pharmacia Biotech AB., Uppsala, Sweden).

The vacuum-dried samples were dissolved in 100 μl of 40mM Li_2CO_3 solution (pH 9.5) and reacted for 35 minutes after adding 200 μl of acetonitril containing 1.5mg/ml dansyl chloride. The reaction was stopped by adding 10 μl of 2% methyl-amine

hydrochloride solution. L-type amino acids were dansylated using the same procedure as Superdex fractions. The dansylated samples were loaded on *u*RPC C2/C18 PC3.2/3 equipped in the Smart system. The components were eluted at a flow rate of 300 μl/min with buffer B (0.1% trifluoroacetic acid (TFA) in H_2O) for the first three minutes, and with buffer C (0.1% TFA in acetonitril) gradually from 0 to 25% for the next 15 minutes and then with buffer C for the last three minutes, all detected by UV monitor at OD_{280nm}.

3. Oocyte Preparation and Microinjection

Four weeks old female mice were superovulated by intraperitoneal injection of 7.5IU pregnant mare serum gonadotropin (Daesung Microbiological Lab., Yeewang, KG. Korea) and followed by a 48-hour later injection of 5IU human chorionic gonadotropin (HCG, Daesung Microbiological Lab., Yeewang, KG. Korea). Cumulus-oocyte complexes (COCs) were recovered 12.5 hours after HCG injection, washed thrice with M_2 and incubated with 150IU/ml hyaluronidase for 3 ~ 5 minutes to remove cumulus cells. The oocytes containing a normal first polar body were selected for microinjection study.

The oocytes were microinjected with 5*pl* of injection buffer S (120mM KCl, 20mM HEPES, pH 7.5) or the buffer S containing Superose fractions (A280nm; 0.230*u*AU for RV1.85, 0.086*u*AU for RV2.10, 0.035*u*AU for RV2.49, 1.870*u*AU for RV 2.73) and Superdex fractions (A245nm; 0.006*u*AU for RV2.00, 0.005*u*AU for RV2.12, 0.003*u*AU for RV2.21) or the buffer S supplemented with glutamic acid (10mM) using a Narishige micromanipulator (Narishige Co. Ltd, Tokyo. Japan) mounted on an inverted microscope (IMT-2; Olympus Optical Co. LTD., Japan). Micro-injected oocytes were incubated in M_{16} (Sigma) at 37℃ for six hours (5% CO_2 in air) and then observed for second PBF.

4. Statistics

The differences in PBF activity among sperm components were statistically analyzed using the chi-square test. A probability of $p<0.05$ was considered statistically significant.

Ⅲ. RESULTS

1. Superose Fractions of Sperm Extract

The sperm extract separated on Superose column included components related to four major peaks at retention volumes (RVs) of 1.85, 2.10, 2.49 and 2.73ml (Fig. 1).

When oocytes were micro-injected with Control, RV1.85 and RV2.10, Superose fraction RV2.10 significantly increased PBF (Table 1; Trial I). However, when oocytes were micro-injected with Control, RV2.49 and RV2.73, the PBF rates were not significantly different among treatments (Table 1; Trial II).

2. Superdex Fractions of Superose Fraction RV 2.10

Superose fraction RV2.10 re-separated on Superdex column had components related to three peaks at RVs of 2.00, 2.12 and 2.21ml (Fig. 2).

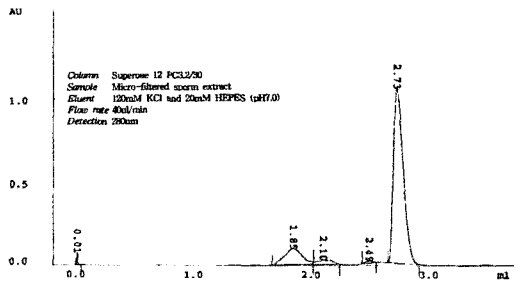


Fig. 1. Superose fractions of sperm extracts. Sperm extract had the components related to four major peaks shown at retention volumes (RVs) of 1.85, 2.10, 2.49 and 2.73 ml.

Table 1. Effect of Superose fractions recovered from sperm extract on second polar body formation (PBF)

Trials	Superose fractions[A] of sperm extract	No. of MⅡ oocytes used	No. (%) oocytes with 2nd PB
I	Control	33	9(27.3)[a]
	RV1.85	26	8(30.8)[a]
	RV2.10	30	15(50.0)[b]
Ⅱ	Control	55	17(30.9)[NS]
	RV2.49	49	13(26.5)
	RV2.73	53	19(35.8)

Superscript A means that MⅡ oocytes were microinjected with HEPES-buffered KCl solution (Control) and with HEPES-buffered KCl solution containing Superose fractions RV1.85, 2.10, 2.49 and 2.73 (RV1.85, RV2.10, RV2.49 and RV2.73), respectively.
Values bearing superscripts a and b were significantly different in Trial I ($p<0.05$).
Superscript NS in TrialⅡ means "no significant difference among treatments" ($p>0.05$).

When oocytes were micro-injected with Control, RV2.00, RV2.12 and RV2.21, Superdex fraction RV2.12 significantly increased PBF (Table 2).

3. Identification of Superdex Fraction RV2.12

Superdex fraction RV2.12 was dansylated and separated by *u*RPC and a component related to a peak retention time (RT) of 6.98 minutes was exhibited (Fig. 3. A). Of L-type amino acids that were dansylated and separated by *u*RPC, only glutamic acid resulted in a peak pattern similar to Superdex fraction RV2.12 (Fig. 3B).

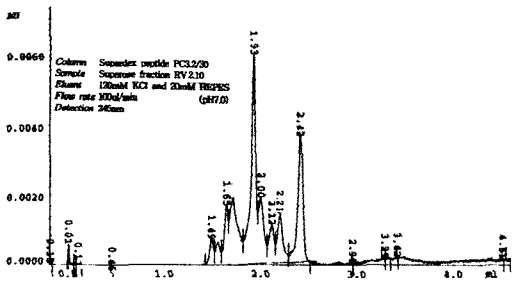


Fig. 2. Superdex fractions of Superose fraction RV2.10. Superose fraction RV2.10 had the components related to three peaks near RV2.10 shown at RVs of 2.00, 2.12 and 2.21 ml.

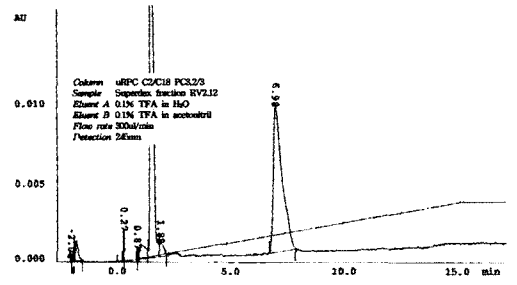
Table 2. Effect of Superdex fractions recovered from Superose RV2.10 on second polar body formation (PBF)

Superdex fractions[A]	No. of MⅡ oocytes used	No. (%) of oocytes with 2nd PB
Control	36	11(30.6)[a]
RV2.02	27	6(22.2)[a]
RV2.12	31	20(64.5)[b]
RV2.21	27	8(29.6)[a]

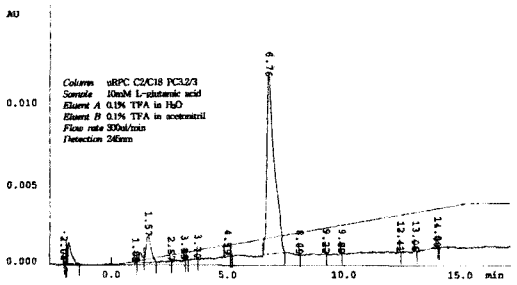
Superscript A means that MⅡ oocytes were microinjected with HEPES-buffered KCl solution (Control) and with HEPES-buffered KCl solution containing Superdex fractions RV2.02, 2.12 and 2.21 (RV2.02, 2.12 and 2.21), respectively.
Values bearing superscripts a and b were significantly different ($p<0.05$).

When 10mM glutamic acid was microinjected into oocytes, it significantly increased PBF more than Control (Table 3).

Ⅳ. ACKNOWLEDGEMENT



(A)



(B)

Fig. 3. uRPC fractionation of Superdex fraction RV2.12 (A) and glutamic acid (B). Superdex fraction RV2.12 and glutamic acid showed peaks at retention times (RTs) of 6.98 and 6.76 minutes, respectively.

Table 3. Effect of glutamic acid on second polar body formation (PBF)

Treatment[A]	No. of MII oocytes used	No.(%) of oocytes with 2nd PB
Control	115	33(28.7)[a]
Glutamic acid	110	60(53.1)[b]

Superscript A means that MII oocytes were micro-injected only HEPES-buffered KCl solution (Control) and with HEPES-buffered KCl solution containing glutamic acid.

Values bearing superscripts a and b in the same column were significantly different ($p<0.05$).

We thank Dr. Clifton A. Baile for critically reading the manuscript.

V. DISCUSSION

When components of ultra-filtrated sperm extract were fractionated on Superose column and the fraction RV2.10 of Superose fractions was micro-injected into MII oocyte, the PBF rates were significantly increased (Table 1; Trial I). In our previous study, similar Superose fractions were obtained in dialyzed sperm extract and Superose fraction RV 2.14 also significantly increased second polar body formation (Kim et al., 2000). According to these results, Superose fraction RV2.10 seemed to include a component(s) for PBF activity. Superose fraction RV2.10 was re-separated with Superdex column and the fraction RV2.12 of Superdex fractions significantly increased PBF (Table 2). This result suggests that Superdex fraction RV2.12 should be a PBF stimulating factor. By *u*RPC analysis, Superdex fraction RV2.12 elucidated to be glutamic acid. Also, Glutamic acid significantly increased PBF by microinjecting into MII oocytes (Table 3). This result demonstrates that glutamic acid may be a functioning type of oocyte activation factor released into oocyte cytoplasm during fertilization.

There are no papers that glutamic acid is a PBF inducing factor or an oocyte activation factor. But, Arena et al. (1992) reported that glutamate activated an inward membrane current. The sperm factor inducing Ca^{2+} oscillation in eggs contains phospholipase C (Parrington et al., 1999). Nakamura et al. (1996) suggested that glutamate stimulated the endogenous PLCx activation by GL_2 alpha of Gq. Sugiyama et al. (1987) reported that a new type of Glu receptor directly activated inositol phospholipid metabolism through interaction with GTP-binding regulatory proteins (Gi or Go), leading to the formation of inositol 1,4,5-trisphosphate ($InsP_3$) and then mobilization of intracellular Ca^{2+}. Considering these results, glutamic acid may stimulate PBF by changing cell membrane potential and/or activating phosphatidyl inositol system for Ca^{2+} transport. In conclusion, glutamic acid should be a PBF inducing

factor of sperm oocyte activation factors.

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(Received October 15, 2002;
Accepted November 10, 2002)