Cloning of boar SPMI gene which is expressed specifically in seminal vesicle and codes for a sperm motility inhibitor protein

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Abstract Boar semen contains a seminal plasma motility inhibitor (SPMI) that blocks the motility of demembranated-reactivated spermatozoa as well as of intact spermatozoa. In this paper, we describe the primary structure of SPMI, the coding of boar SPMI cDNA gene and its expression in various porcine tissues. Nucleotide sequence analysis of the 645-bp SPMI cDNA predicts a coded polypeptide of 137 amino acid residues which includes a 21-residue signal peptide and a 116-residue secreted protein. The amino acid sequence of SPMI was found to be highly homologous to AQN-3, a member of spermadhesin family proteins of boar that bind to spermatozoa. Expression of the boar SPMI gene detected by Northern blot analysis revealed that its expression is very abundant in seminal vesicles and specific to this tissue.

Key words: Boar SPMI; Seminal plasma sperm motility inhibitor; Seminal vesicle protein; Seminal plasma; Demembranated-reactivated spermatozoa; Spermadhesin

1. Introduction

Seminal plasma contains factors that affect sperm motility. While secretions from the seminal vesicles are generally considered deleterious, those of the prostate have beneficial effects [1,2]. The seminal plasma of all mammals investigated so far contains a factor capable of inhibiting the motility of demembranated-reactivated spermatozoa [3–5]. This seminal plasma motility inhibitor (SPMI) was first purified from human seminal plasma where its molecular weight has been estimated at 15–18 kDa [6]. Purified human SPMI has also been shown to inhibit the motility of intact spermatozoa when incubated at an adequate concentration [7]. SPMI has recently been purified from boar seminal plasma where its molecular mass has been estimated as 50 kDa by molecular sieving, but three peptides of 14, 16 and 18 kDa were detected after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) run under reducing conditions [8]. Immunohistological staining with antibodies against boar SPMI revealed that this factor was mainly detected on the plasma membrane of epithelial secretory cells bordering the lumen of seminal vesicles [9].

To better understand the structure and function of boar SPMI, we have analysed the N-terminal amino acid sequences of the three peptides composing this factor and cloned the cDNA gene. We also have studied the expression of this boar SPMI gene in various male and female porcine tissues.

2. Materials and methods

2.1. Semen collection and seminal plasma storage

Boar semen was collected by masturbation and provided by the Kanagawa Prefectural Live Stock Experiment Station (Ebina, Japan). Semen samples were centrifuged at $10,000 \times g$ for 15 min at 20°C to eliminate any cellular elements and debris. Following centrifugation, the clear seminal plasma was collected and stored at −70°C until used.

2.2. Reproductive tract organ, urinary bladder, liver, spleen, ovary, oviduct, uterus, vagina from boar and seminal vesicle from human

Boar seminal vesicle, prostate, testis, epididymis, bulbourethral gland, kidney, urinary bladder, liver, spleen, ovary, oviduct, uterus and vagina for preparation of mRNA were collected from freshly slaughtered animals (Kanagawa Food and Meat Inspector, Sagamihara, Japan). Human seminal vesicles were obtained from patients undergoing radical cystectomy and prostatectomy. These organ were cut into small pieces and immediately frozen by dry ice and stored −80°C until used.

2.3. Purification procedures of boar SPMI

The purification procedure included the dialysis of seminal plasma against 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 mM DTT and the separation by column chromatographies on SP-Sephadex C-25 and Phenyl-Sephalose CL-4B as described in details by Iwamoto et al. [8]. By these procedures, SPMI preparation was highly purified with a 18% recovery of inhibitory activity. The molecular mass of SPMI in native conditions has been estimated as 50 kDa by molecular sieving, but three polypeptides with 14, 16 and 18 kDa were observed by SDS-PAGE under reducing conditions.

2.4. N-terminal sequencing

Purified SPMI was resolved into three bands by electrophoresis in 12.5% polyacrylamide gel in the presence of 0.1% SDS and 5% β-mercaptoethanol, electroblotted onto PVDF (polyvinylidene difluoride) membrane and stained with Coomassie Brilliant Blue G. Each band was excised and analysed for the N-terminal sequence by Applied Biosystem Analyser.

2.5. Preparation of RNA

Individual tissues (~5 g) were homogenized in 20 ml of GIT buffer (4 M guanidine isothiocianate, 0.7% β-mercaptoethanol, 25 mM sodium citrate, pH 7.0) and then sarcosyl was added to a final concentration of 0.5%. The homogenate was layered onto a 20-ml cesium chloride cushion (5.8 M CsCl, 10 mM EDTA, pH 7.0) and the RNA was pelleted by centrifugation at 25,000 rpm for 24 h in a Beckman SW28 rotor. The pellet was washed once with 70% EtOH, dried and dissolved in distilled water.

Poly(A)-containing RNA was purified from the total RNA by affinity binding to Oligotex (dT)30 (Takara, Osaka, Japan) according to the protocol provided by manufacturer.

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2.6. Construction and characterization of cDNA libraries and cloning

The boar seminal vesicle cDNA library was constructed from 5 μg of poly(A)$^+$RNA using Zap cDNA Synthesis Kit (Strategene, La Jolla, CA). First-strand DNA was synthesized using Mo-MuLV reverse transcriptase, a linker primer and nucleotide mixture. The second-strand synthesis was done by RNase H and DNA polymerase 1 in the presence of all four nucleotides. After cDNA synthesis, the resulting cDNAs was blunt-ended by an incubation with Klenow flagment of T4 DNA polymerase. The boar seminal vesicle cDNA library was constructed by insertion of cDNA into pUC19 at the *SmaI* site and then transfected to the *E. coli* JM 109 strain. Gene cloning for SPMI-specific cDNA was carried out according to the method described by Senda et al. [10].

2.7. Northern blot analysis

Denatured RNA sample (15 μg) were separated by electrophoresis in 1.0% agarose gels containing 2.7% formaldehyde. RNAs were transferred to Hybond N membrane (Amersham, UK) by capillary blotting for 5 h in 20 × SSC (3 M NaCl, 0.3 M sodium citrate). Northern blots were rinsed, dried and cross-linked by UV irradiation. Prehybridization was performed for 4 h at 42°C in a solution containing 50% formamide, 1 M sodium chloride, 1.0% SDS and 25 μg/ml salmon sperm DNA. Blots were hybridized overnight at 42°C with ^{32}P-labeled DNA-probe representing whole coding region of SPMI cDNA in prehybridization mixture. The blots were washed successively in 2 × SSC/0.1% SDS, 0.1 × SSC/0.1% SDS 2 × at 60°C, then exposed to X-ray film for 3–24 h.

3. Results and discussion

3.1. Heterogeneity in amino acid sequence of SPMI components

The boar SPMI protein was purified as described in Materials and Methods. These polypeptides (14, 16 and 18 kDa) that constitute the intact SPMI protein were then separated by SDS-PAGE under reducing conditions and analysed for their N-terminal amino acid sequences. As shown in Table 1, all three polypeptides (14, 16 and 18 kDa) contained rather similar N-terminal amino acid sequences. In addition, each polypeptide band contained at least two species of polypeptides which were distinct from each other by one or two amino acid substitutions. These results suggested that the boar SPMI may consist of at least six heterologous polypeptides, resulting in the occurrence of multiple isozymes.

3.2. Molecular cloning of cDNA encoding one of boar SPMI components

To understand the complexity of the N-terminal sequence of SPMI components and to clarify its overall structure, we have constructed a cDNA library and cloned a cDNA gene that codes for one of the SPMI components. Cloning was done by PCR mediated amplification of cDNA gene present in the library by the 5′-side primer designed from the N-terminal amino acid sequence and the adapter primer that binds to the 3′-end of SPMI mRNA. The 5′-side primer (35 mer) contained the sequence of ACT AAT GGA ATT CCT CA(A,C) AA(C,T) AA(A,G) GG(C,T) TC(A,G,C,T) GA(C,T) GA with a degeneracy of 128. The 3′-side primer (42 mer) contained a sequence of AAG ACG CCT TGA AAT CTA GAG CTA ATT TTT TTT TTT TTT TTT. After 30 cycles of PCR reaction with a template consisting of cDNA prepared from the seminal vesicle RNA, a band of ~600 bp was obtained which then was ^{32}P-labeled and used as a probe to obtain the SPMI specific clone from cDNA library. The clones that hybridized with this ^{32}P-labeled probe were characterized extensively. One clone was found to contain 645-bp insert which harbored the potential protein synthesis initiation signal (ATG), a long open reading frame (116 amino acids) and 3′-polyA signal (Fig. 1). The open reading frame possessed an identical amino acid sequence (residues 22–46) to that detected by protein analysis in the 14-kDa SPMI component (Table 1). The cloned SPMI cDNA can encode a polypeptide (precursor protein) of 137 amino acid residues starting with methionine at amino acid residue −21 position. However, since none of the six polypeptides ranging from 14 to 18 kDa (Table 1) contained the first 21-amino acid stretch, the upstream peptide with 2one amino acid may represent the signal peptide which is cleaved off upon secretion from the seminal vesicular epithelium. Indeed, Kwok et al. [11] have recently described PSP-I and -II genes that code for porcine seminal proteins, which represented only 50% homology in amino acid sequence to the mature SPMI but showed as high as 98% homology to this putative signal peptide of 21 amino acids. Thus, the first 21 residues may be characteristic of signal peptides of secretary proteins of porcine origin. The predicted amino acid sequence indicates that the mature form of SPMI component is a basic protein of molecular weight 12,878 Da with a net positive charge of +4, in agreement of our previous observation that the isoelectric point (pI) of SPMI is 8.7–9.1 [9].

Table 1
N-terminal amino acid sequences of purified boar SPMI polypeptides

Lower band (14 kDa)	AQNKGSDD-GGFLKNYSGNISYYK	(80%)
	W	(20%)
Middle band (16 kDa)	AQNKGSDSLGGVLK	(70%)
	F F	(30%)
Upper band (18 kDa)	AQNKGSDD-GGVLK	(50%)
	P	(50%)

Purified boar SPMI was separated by SDS-PAGE under reducing conditions into three bands of 14, 16 and 18 kDa. Each of the three bands contained two peptides. For the second peptide of each band, only the amino acids that were different from the predominant peptide were indicated.

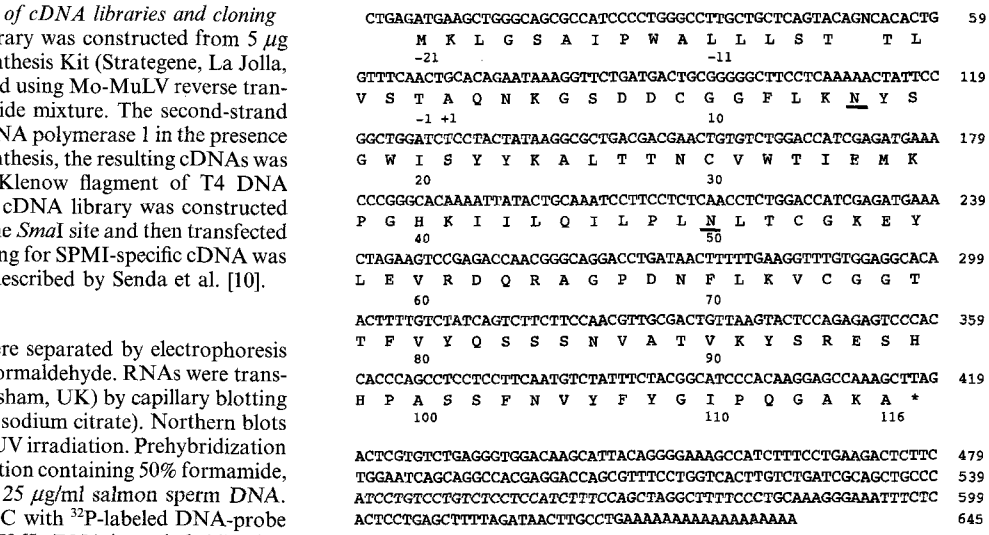

Fig. 1. Nucleotide sequence of boar SPMI cDNA gene. The complete sequence is presented in the 5′ to 3′ direction. The numbering of the nucleotide sequence is shown at the right-hand side, and that of the amino acid sequence is shown below the amino acid residues. Negative numbers correspond to amino acid residues in the signal peptide and positive numbers refer to amino acid residues of the predicted secreted proteins. The underlined asparagine residue is the glycosylated residue.

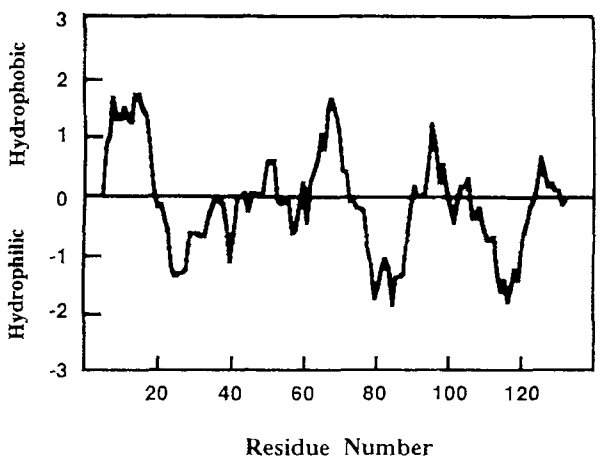

Fig. 2. Hydropathy plots of boar SPMI. The plots were determined by using the program of Kyte and Doolittle [22]. Hydrophobic regions are shown as an upward excursion from the x axis, whereas hydrophilic regions are shown as a downward excursion from the x axis.

Two protein N-linked glycosylation sites, N^{15}-Y-S and N^{50}-L-T, were found in the SPMI molecule. These glycosylation sites were also found in the AQN-3, one of the boar spermadhesin family proteins (Table 2), at the same positions [12]. The amino sugar analysis of AQN-3 by fast-atom-bombardement mass spectrometry revealed that the N^{50}-L-T contained a single oligosaccharide chain, while the N^{15}-Y-S did not [12]. The small discrepancy between the apparent molecular mass (14 kDa) of SPMI and the predicted molecular mass (12,878 Da) may thus suggest that the boar SPMI also contains N-linked sugar at these sites. The hydropathy profile of the predicted SPMI protein suggests that the boar SPMI is a rather hydrophilic protein (Fig. 2).

3.3. Comparison of amino acid sequence between AQN-3 [12,13] and SPMI protein predicted by cloned cDNA sequence

Fig. 3 shows that AQN-3 and SPMI are very homologous in amino acid sequence with a homology index of 97%. There are only two differences; one at residue 78 (T/SPMI vs. G/AQN-3) and another at residue 95 (E/SPMI vs. D/AQN-3). The latter alteration is a homologous substitution from glutamic acid to aspartic acid implying that the presence of negatively charged amino acid may be important in the biological function of both of these proteins. The third difference in the residue 85 can not be discussed because of unavailability of data concerning the identity of X in AQN-3. However, Sanz et al. [13] speculated that X could be methyl histidine based on the data with mass spectrum analysis.

3.4. Expression of SPMI gene in various porcine organs

The levels of SPMI gene expression were determined by Northern blot analysis using the probe DNA encoding the open reading frame of the cloned SPMI cDNA gene. It was soon noted that the boar seminal vesicle is extraordinarily abundant in SPMI mRNA (Fig. 4, lanes A,B). This observation was supported by other results. When the first-strand cDNA was made from the total RNA extracted from seminal vesicle, a distinct cDNA band reverse transcribed from SPMI mRNA represented a major population, with very few diffused cDNA bands from other mRNA species. It was thus be assumed that more than 50% of total mRNA in seminal vesicle could be SPMI mRNA. In contrast, there was no detectable level of SPMI expression in the testis, epididymis, bulbourethral gland, urinary bladder, liver, spleen as well as in the reproductive organs of the sow (Fig. 4, lanes C–G). The only exception was the prostate, where a faint band was observed but only after more than 24 h of exposure to hybridization with the poly(A)$^+$ RNA or with a total RNA. We speculate, however, that this faint band might have stemmed from the contamination of epithelial cells of seminal vesicle origin into prostate tissue. Anatomically, the bilateral ejaculatory duct which always contain the fluid from seminal vesicles penetrates into the prostate and {11}reaches the urethra at verumontanum. Thus, it would appear that the expression of the SPMI gene is highly specific to the seminal vesicles. When the RNAs from human seminal vesicles were tested, no sign of hybridization was observed for six individuals tested (Fig. 4, lane H). These results suggest that there is no homology between human and boar SPMI sequence.

3.5. Biological roles of SPMI

SPMI have been found in all seminal plasma investigated so far [3–5]. However, these SPMI are quite different from each other. Although boar and human SPMI have similar basic pI [6,9], the two molecules are structurally dissimilar. In the porcine species, SPMI has a molecular weight of 50 kDa in native conditions being made up of three stable subunits of 14, 16 and 18 kDa. In contrast, the human SPMI exists as a 52-kDa polypeptide immediately after ejaculation, but is rapidly degraded

Table 2
Proteins in the boar spermadhesin family

Name	Source	Location	Size of protein (a.a. residue or mol.wt.)	Function	Reference
SPMI	S.P.$^+$	Seminal vesicle	116 a.a.	Sperm motility inhibition	Present paper
PSP-I(SP3/SP6)	S.P.	Seminal vesicle	112 a.a.	n.d.	[11]
PSP-II(SP4/SP7)	S.P.	Seminal vesicle	116 a.a.	n.d.	[11]
AWN	e.sperm*	Seminal vesicle	112 a.a.	ZP-binding	[17]
AQN-1	S.P.	n.d.**	n.d.	Sperm-ZP recognition	[16,20]
AQN-2	e.sperm	n.d.	18–20 kDa	ZP-binding	[12,20]
AQN-3	e.sperm	n.d.	116 a.a.	Carbohydrate binding	[12,13]
ACR-3 antigen (or EJa)	e.sperm	n.d.	17–18 kDa	ZP-binding	[18]
EJc–f	e.sperm	n.d.	n.d.	ZP-binding	[19]

$^+$Seminal plasma; *ejaculated sperm; **not defined.

by proteases from the prostate into a less active peptide of 15–20 kDa [14]. Despite these differences, both human SPMI [7] and boar SPMI (unpubl. data) block the motility of demembranated-reactivated spermatozoa as well as intact spermatozoa of both origins. On demembranated-reactivated spermatozoa, SPMI inhibits dynein ATPases, the key enzymes involved in flagellar motility [6]. On intact spermatozoa, SPMI action is associated with a decrease in beat/cross-frequency and velocity while having no effect on the linearity of sperm trajectories [7].

A series of small molecular weight proteins have recently been reported in porcine seminal plasma [11–13,15] and collectively called spermadhesins. These molecules bind to spermatozoa where they have been suggested to act as decapacitation factors or acrosomal binding factors while different members of this family of sperm binding proteins may act as capacitation factors via their heparin binding properties or as primary receptors for zona pellucida glycoproteins [15].

The spermadhesin family presently includes 11 members: PSP-1 (or SP3/SP6), PSP-2 (or SP4/SP7) [11], AWN, AQN-1, AQN-2, AQN-3 [12,13,16–17,20], ACR-3 antigen (or Eja) [18], EJc, EJd, EJe and EJf [19]. The present data clearly indicate that, because of its 97% similarity to AQN-3 peptide, boar SPMI should became the 12th member reported of this family (Table 2). No homolog of boar spermadhesin has yet been reported in other mammals. However, the observation that a SPMI is also present in seminal plasma of humans raises the possibility that some amino acid sequence may be conserved between boar and human SPMI. A zona pellucida binding protein of size similar to that of spermadhesins but different in primary structure from spermadhesins has recently been isolated from rabbit testis [21]. Further studies should shed light on whether the motility inhibitory activity associated with boar SPMI gene product may represent a new property of associated spermadhesin molecules.

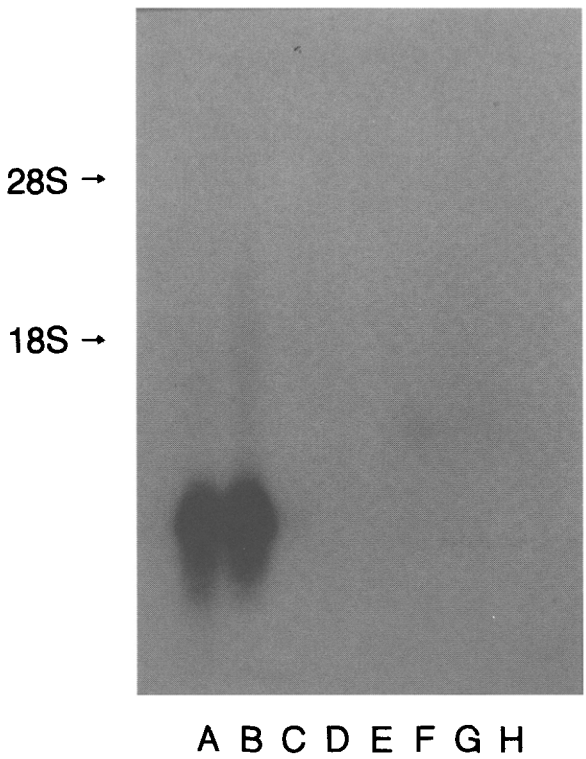

Fig. 4. Northern blot analysis. 15 μm of RNA from organ tissues was separated in a 1.0% agarose gel, blotted to Hybond N membrane and hybridized with ^{32}P-labeled DNA probe representing the whole coding region of SPMI cDNA. Membranes were exposed to X-ray film for 3 h. Lanes A,B, seminal vesicles from two boars; lane C, boar prostate; lane D, boar testis; lane E, boar epididymis; lane F, boar kidney; lane G, boar liver; lane H, human seminal vesicle. Arrows indicate positions of 28S and 18S rRNA.

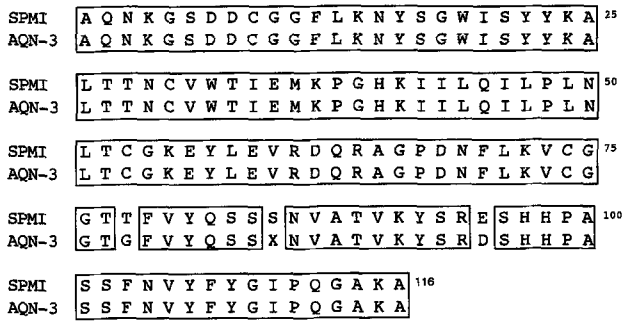

Fig. 3. Comparison of amino acid sequence predicted from the cloned boar SPMI cDNA gene with AQN-3 protein. Identical residues are shown in boxes.

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