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Secretory Origin and Temporal Appearance of the Porcine β-Microseminoprotein (Sperm Motility Inhibitor) in the Boar Reproductive System

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ABSTRACT A specific antiserum against the porcine sperm motility inhibitor (SMI) was used in Western blotting analysis of tissue homogenates to reveal the possible origin of SMI in the boar reproductive system at different ages. The ages of the boar used were day 0, day 15, day 30, day 60, day 100, day 120, day 135, day 150, and day 210. The tissue homogenates of the day 60 and older showed immunoreaction. The results were further checked by indirect immunohistochemical staining and observed under light microscope. The SMI antigen appeared in the epithelial cells and in the lumen of the secretory ducts of the prostate gland. These results indicate that porcine SMI is synthesized only by the postnatal prostate gland. The homogenate of the prostate gland of day 100 was also used for the purification of SMI. The prostatic SMI was co-eluted with the seminal SMI in the reversed phase HPLC. Mass spectrometric analysis of the prostatic SMI revealed a molecular weight of 10,066. These results indicate that the prostatic SMI is identical to that purified from seminal plasma (Jeng et al., 1993; Biochem Biophys Res Communi 191:435–440). *Mol. Reprod. Dev. 58:63–68, 2001.* © 2001 Wiley-Liss, Inc.

Key Words: sperm motility inhibitor; prostate gland; Western blotting; immunohistochemical staining

INTRODUCTION

Most noncellular elements of ejaculated semen are secreted by accessory sexual glands, which comprise the seminal vesicle, the prostate gland, and the bulbourethral gland. The seminal plasma is the common secretion of accessory sexual glands and contains a wide variety of enzymatic and nonenzymatic proteins (Mann and Lutwak-Mann, 1981). A sperm motility inhibitor (SMI)[1] purified from porcine seminal plasma has been shown to inhibit the sperm motility in a reversible and dose-dependent manner as previously reported (Jeng et al., 1993). The amino acid sequence of the peptide fragments of SMI was determined and compared with porcine β-microseminoprotein (Fernlund et al., 1994). The agreement in the peptide sequences and molecular masses led us to conclude that SMI is identical to β-microseminoprotein[1] (Chao et al., 1996). The human analog, β-microseminoprotein, also called PSP 94 (a prostatic secretory protein of 94 amino acids), is an unglycosylated protein with an unknown function (Green et al., 1990). Porcine SMI is a single-chain protein of 91 amino acids and has a deletion of three amino acids in the N-terminal region, but otherwise manifests similarity in amino acid sequence including conservation of 10 cysteine residues of β-microseminoprotein (Fernlund et al., 1994).

Iwamoto et al. (1992) purified a motility-dynein ATPase inhibitor, named SPMI, from boar seminal plasma. Nucleotide sequence analysis of the 645-bp SPMI cDNA predicted a coded polypeptide of 137 amino acid residues, which includes a 21-residue signal peptide and a 116-residue secreted protein. SPMI is secreted by seminal vesicle (Iwamoto et al., 1993, 1995). Human seminal plasma also contains SPMI originating from the seminal vesicles as a 52 kDa precursor form (Robert and Gagnon, 1995). Both SPMI and SMI have motility inhibitory effect on porcine sperm. However, they are not the same protein.

Our previous studies have shown that porcine SMI can inhibit the Na^+, K^+-ATPase and reduce the sperm motility reversibly (Jeng et al., 1993; Chao et al., 1996).

[1]Since the sperm motility inhibitor (SMI) was found to be identical to β-microseminoprotein, these two terms are used interchangeably in this report.

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The human β-microseminoprotein was reported to be present in several mucus-associated tissues other than the reproductive organs (Weiber et al, 1990), which suggested a possible functional role in reproductive as well as nonreproductive tissues. It is of great interest to find out whether porcine β-microseminoprotein has the same distribution profile with its human analog because this information would shed some light on the problem of its biological functions. We, therefore, investigated the secretory origin of porcine β-microseminoprotein using Western blotting and immunohistochemical staining techniques. In this report, we conclude that porcine β-microseminoprotein is synthesized only by the postnatal prostate and secreted into the seminal plasma.

MATERIALS AND METHODS

Semen Collection and Purification of Porcine β-Microseminoprotein

The fertile boars of the MeiShan strain were used. Freshly ejaculated semen (sperm-rich fraction) was collected by the gloved-hand method into a warmed beaker and filtered through three layers of cheesecloth. The filtrate was centrifuged at 600*g* for 30 min at 4°C. The supernatant was dialyzed overnight at 4°C against three changes of double deionized water, lyophilized, and stored at −70°C until used. The porcine β-microseminoprotein was purified from seminal plasma as previously described (Jeng et al., 1993).

Antibody Induction

A male New Zealand rabbit was used. Intrasplenic immunization was performed as previously described (Hong et al., 1989). Emulsion was prepared by mixing saline containing 0.3 mg of antigen (SMI1) with Freund's complete adjuvant (Gibco). Two weeks after immunization, the serum titer was checked by double diffusion and solid-phase enzyme-linked immunosorbent assay (ELISA). Optical densities were read in a microplate reader (model 450, Bio-Rad). The sera with high titer were collected, aliquoted, and stored at −20°C.

Western Blotting Analysis

The assay of different tissue homogenates. The homogenates of porcine male reproductive organs including seminal vesicle, prostate gland, bulbourethral gland, epididymis, and testis, were prepared with 3 volumes (w/v) of extraction buffer (50 mM Tris–HCl, pH 8.5, 5 mM EDTA, 100 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin) at 4°C (Kwok et al., 1993). The homogenates were then centrifuged twice at 4°C for 20 min at 20,000*g*. The supernatants were analyzed by 8–20% tricine gradient SDS–polyacrylamide gel electrophoresis (SDS-PAGE, Novex Experimental Technology) (Laemmli, 1970) and then transferred to a polyvinylidene difluoride membrane (PVDF, Immobilan-P; Millipore Corporation, Bedford). The PVDF blot was blocked with 5% skimmed milk in blocking buffer (0.05 M Tris–HCl, 0.9% NaCl, pH 7.6) in 4°C overnight. The primary antibody (the antiserum against SMI, 1500 × dilution) was then incubated with the PVDF blot for 1 hr at room temperature. After washing with TBST (Tris-buffered saline Tween buffer), 0.5 M Tris–HCl, the secondary antibody (alkaline phosphatase conjugated antibody against rabbit IgG, 2500 × dilution in blocking buffer) for 1 hr. The blot was then washed and rinsed with alkaline phosphatase buffer for 5 min. The NBT (4-nitro blue tatraedium chloride) and BCIP (5-brom-4-chlor-3-indolyl-phosphate) were added for staining. The stop solution (0.1 M Tris, 0.01M EDTA, pH 8.0) was used to stop the reaction. The blot was then air-dried. Some homogenates of other organs including trachea, stomach, duodenum, jejunum, ileum, liver, and spleen were also prepared for detecting whether they had SMI immunoreaction or not.

The assay of prostatic homogenates of different ages. The ages of the boar used were day 0, day 15, day 30, day 60, day 100, day 120, day 135, day 150, and day 210. The prostatic homogenates were applied to Western blotting analysis according to the methods described above.

Indirect Immunohistochemical Staining

Specimens were prepared by paraffin sectioning (Minotome, IEC) of the prostate gland, bulbourethral glands, seminal vesicles, epididymis, testis, trachea, stomach, duodenum, jejunum, ileum, liver, and spleen. The procedures given in the manual of the ABC detection system (DRAFT, level1, multispecies Avidin–Biotin Complex detection system; Signet Laboratories, Inc.) were followed except that some incubation times were modified. The counter-stain with hematoxylin was done. The slides were dehydrated and mounted. The control sections were treated in the same manner except that normal rabbit serum was added instead of the antiserum. These sections were observed under a microscope and photographed.

Purification of the Porcine β-Microseminoprotein From the Prostate Gland

Porcine β-microseminoprotein was purified from the homogenate of the porcine prostate gland of 100-day-old pigs according to the method for the purification of SMI from seminal plasma (Jeng et al., 1993).

Mass Spectrometry

Porcine β-microseminoprotein purified from porcine prostate gland was dissolved in 50% acetonitrile containing 0.1% acetic acid to make a final concentration of 10 μM and analyzed in an API-100 mass spectrometer (PE SCIEX) at an infusion rate of 5 μ1/min. The spectra were analyzed by the software (BioTool Box) supplied by the manufacturer.

RESULTS

Immunohistochemical studies and Western blotting indicated that porcine β-microseminoprotein did not

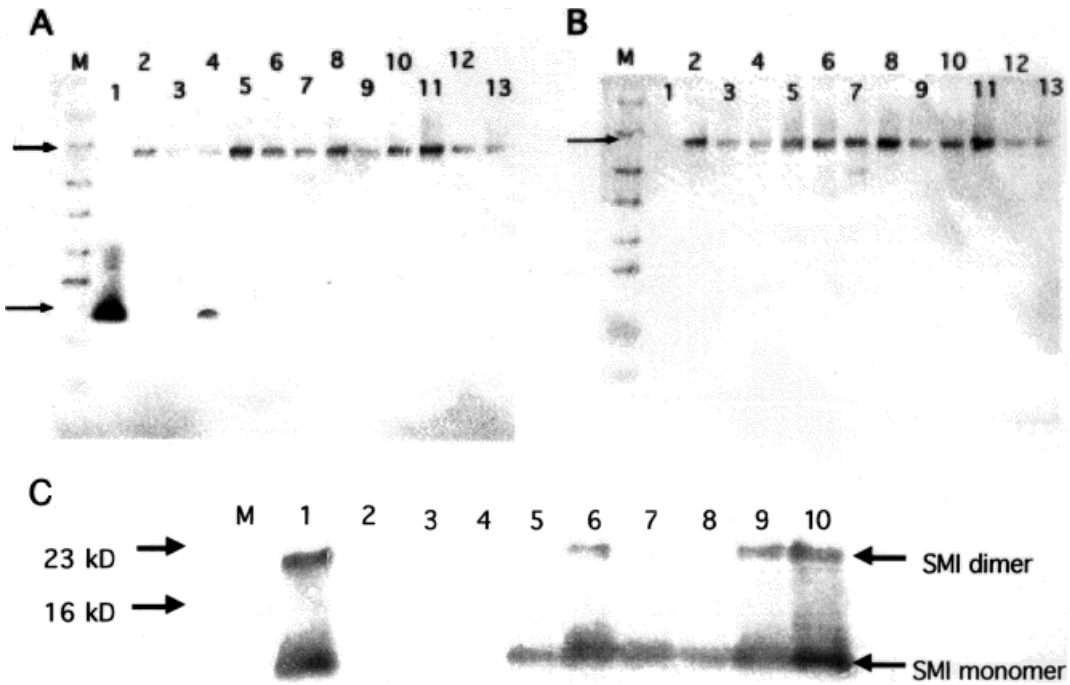

Fig. 1. Western blotting of tissue homogenates (**A**, **B**) and temporal appearance of SMI in prostate (**C**) using immunoreaction with antiserum against SMI. A, B: M: molecular weight marker, 1: seminal SMI, 2: seminal vesicle, 3: bulbourethral glands, 4: prostate gland, 5: epididymis, 6: testis, 7: stomach, 8: duodenum, 9: jejunum, 10: ileum, 11: trachea, 12: spleen, and 13: liver. Each lane is loaded with an amount of 3 μg of total protein, except the lane of SMI which is 1 μg. The age is 100 days. A is the experimental group; B is the control group using normal rabbit serum. The upper arrows indicate the nonspecific band of 52 kDa. The lower arrows show the position of SMI. C: temporal expression of SMI. M: molecular weight marker, 1: SMI, 2: prostatic homogenate of day 0, 3: day 15, 4: day 30, 5: day 60, 6: day 100, 7: day 120, 8: day 135, 9: day 150, 10: day 210.

appear in the prostate tissue before day 30 (Fig. 1C). At day 60 and older, it appeared only in the prostate gland (Figs. 1 and 2).

The western blotting analysis of tissue extracts (Figs. 1A, B) showed that the prostate gland is the only origin for the synthesis of porcine β-microseminoprotein. All the other tissues such as seminal vesicle, bulbourethral glands, prostate, epididymis, testis, stomach, duodenum, jejunum, ileum, trachea, spleen, and liver did not show any immunoreaction with the SMI antiserum.

The Western blotting analysis of prostatic extracts (Fig. 1C) showed that the prostate glands older than 60 days have immunoreaction with the SMI antiserum. The upper band is very likely the SMI dimer based on its immunoreaction and the molecular weight.

The secretory origin of porcine β-microseminoprotein is also confirmed as shown in Fig. 2 by light microscopy using the immunohistochemical localization method. The staining was located in the cytoplasm of the epithelial cell and within the secretory duct of prostate gland as brown pigments. The control sections incubated with normal rabbit serum show no staining except some light background in the epithelium and the seromucous gland in the submucosa of the trachea (data not shown).

Figure 3 shows the HPLC profile for the purification of porcine β-microseminoprotein. It reveals that porcine β-microseminoprotein is the major protein component of prostate gland. The molecular weight was found to be 10,066, as determined by mass spectrometry (Fig. 4).

DISCUSSION

In our previous study (Chao et al., 1996) we found that porcine SMI is identical to β-microseminoprotein. Its human analog has been characterized in man and the ape and is one of the predominant proteins in the secretion of the human prostate gland (Lilja and Abrahamsson, 1988). The human β-microseminoprotein has been found to be present in several nonprostatic tissues by immunohistochemical staining (Weiber et al., 1990) and Northern blot analysis (Ulvsbäck et al., 1989). Its distribution profile seems to suggest some association with mucus secretion (Fernlund et al., 1996).

In the present study we found that porcine SMI was clearly detected by immunohistochemical staining of the prostate gland (Fig. 2). Other nonprostatic tissues including the seminal vesicle, bulbourethral gland, testis, and epididymis of the reproductive system; the stomach, duodenum, jejunum, and ileum of the digestive system and the trachea of the respiratory system showed very little immunoreaction. The Western blotting analysis revealed an immunoreactive band of about 52 kDa in all tissues examined which obviously is

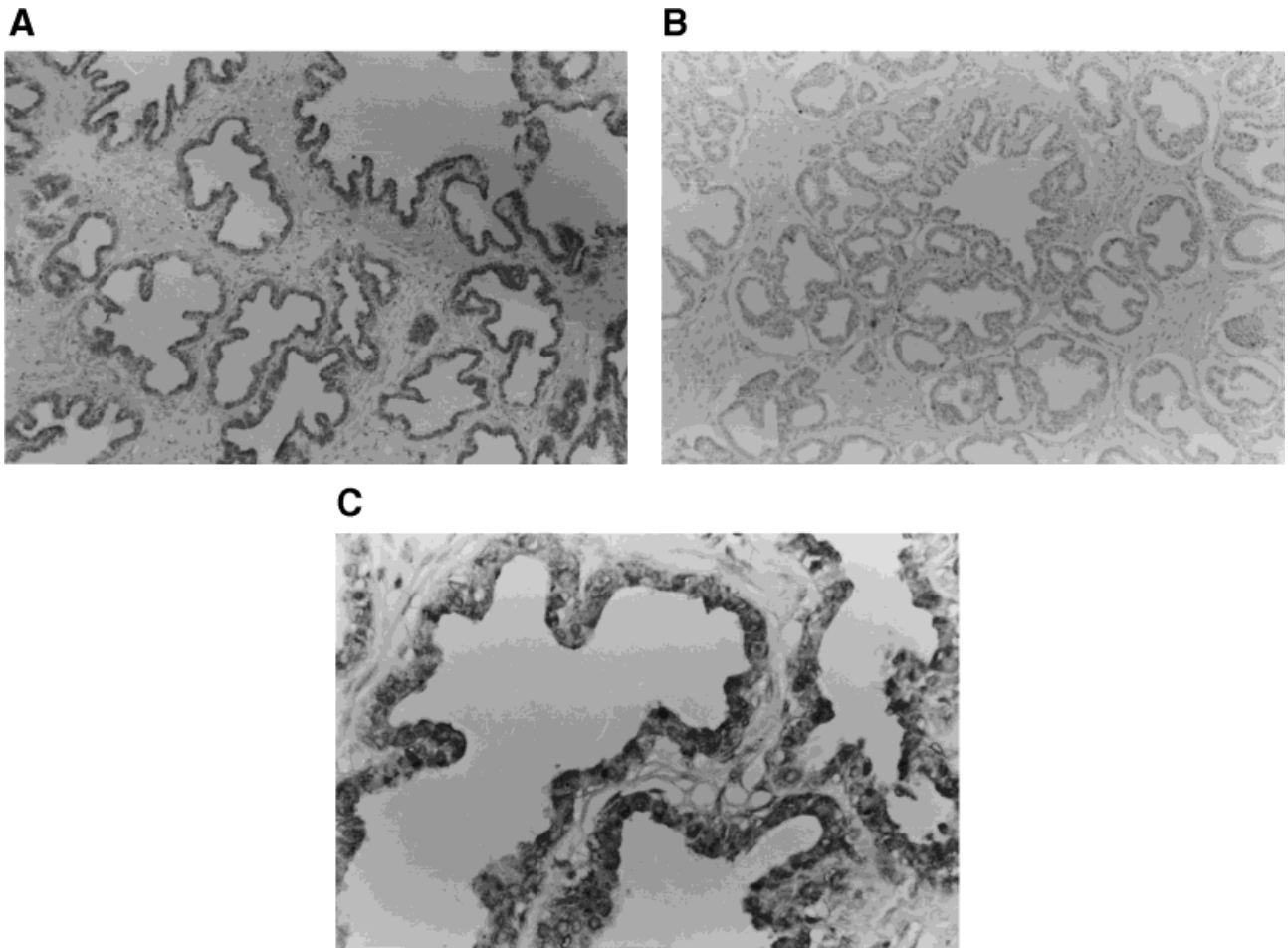

Fig. 2. A: Immunohistochemical localization of SMI in prostate gland of day 100 under a light microscope. **B**: Control group. **C**: Magnification of A. The SMI antigen is stained as brown particles in the cytoplasm of the epithelial cell within the secretory duct. A and B are × 100, C is × 400. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

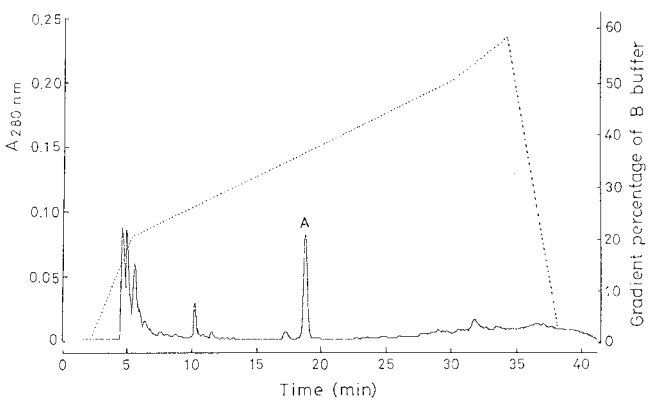

Fig. 3. HPLC profile of prostate extract through an Aquapore RP-300 column. **A**: 0.07% trifluoroacetic acid in water; **B**: 0.07% trifluoroacetic acid in acetonitrile. The concentration of B increased from 20% to 60% within 30 min. Peak A is the prostatic SMI. The prostatic SMI was co-eluted with the seminal SMI in the reversed phase HPLC. Mass spectrometric analysis of the prostatic SMI revealed a molecular weight of 10,066.

not related to SMI. This immunoreactive band was also observed in the control group stained with normal serum (Fig. 1A,B). It is likely that some unknown proteins that could recognize the serum components (probably the immunoglobulins) were responsible for this nonspecific immunobinding. This positive reaction is in agreement with a very weak staining in the control group treated with normal rabbit serum (Fig. 2). The presence of β-microseminoprotein in the trachea of the pig as reported by Fernlund et al. (1994) could be due to this nonspecific immunoreaction.

The present report reveals that the human and porcine β-microseminoproteins, although homologous in sequence, are so different in its tissue distribution profile. There is no doubt that β-microseminoprotein is a prostatic protein and must have some functions in the reproductive system. Our previous report suggested one possible function of β-microseminoprotein; that is, to reversibly and mildly reduce the sperm motility which can be restored by the female follicular fluid

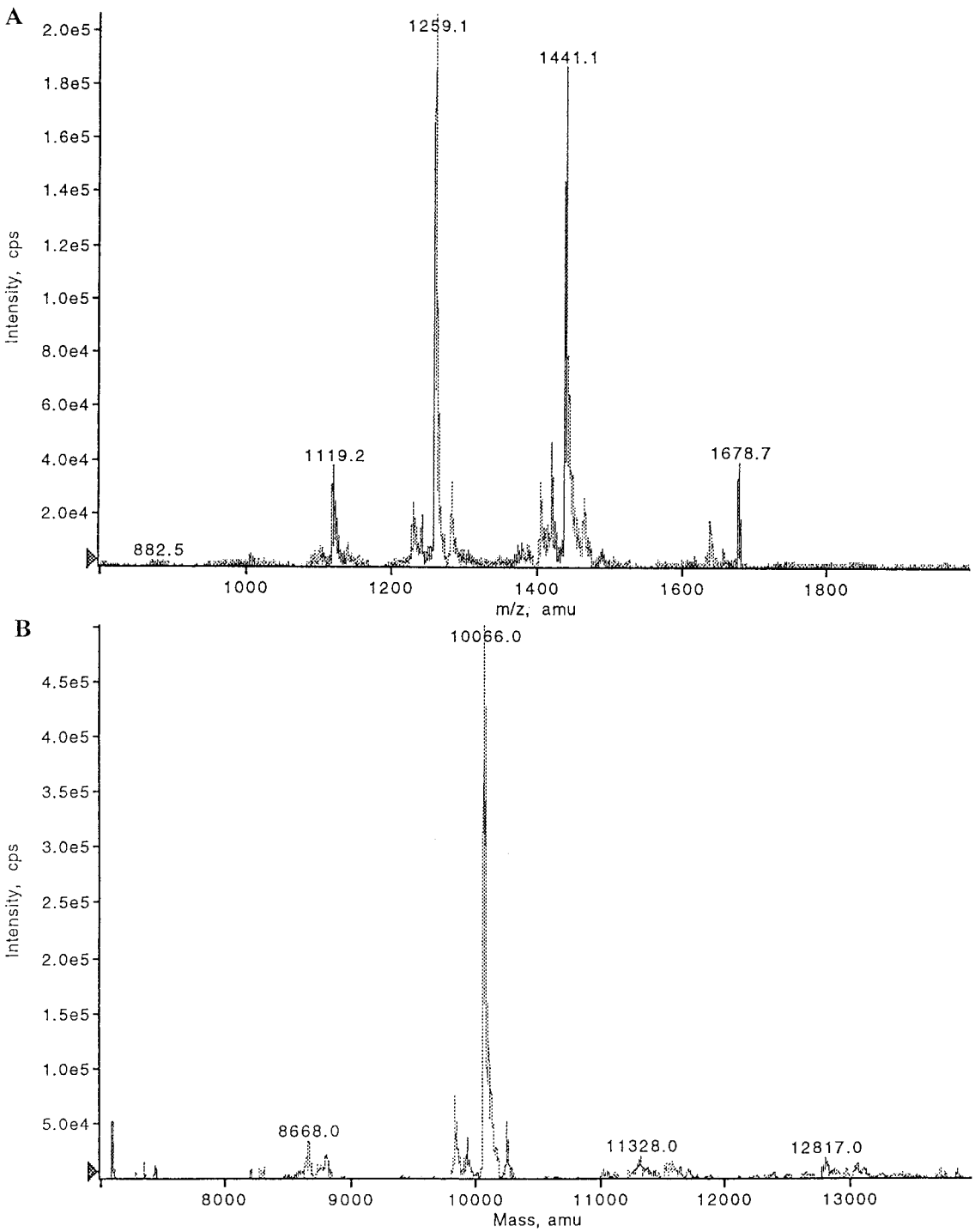

Fig. 4. Electrospray mass spectrum of the SMI purified from porcine prostate gland. **A**: Original spectrum. **B**: Computer deconvolution of the peaks in A. The theoretical value derived from protein sequence of SMI is 10,068 Da (Fernlund et al., 1994; Chao et al., 1996).

(Jeng et al., 1993). It makes sense that the sperm motility is temporarily inhibited before entering the female reproductive tract. The fact that the mature spermatids appear in the boar of MeiShan strain at 60 days of age (Cheng, 1983) is in agreement with our present finding that SMI is not synthesized until this age. All these suggest that the expression of SMI may be coordinated with the course of sexual maturation. Of course, further investigations are needed to clarify this point.

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