

# Ejaculates from the Common Marmoset (*Callithrix jacchus*) Contain Semenogelin and Beta-Microseminoprotein but not Prostate-Specific Antigen

CAMILLA VALTONEN-ANDRÉ,<sup>1\*</sup> A. YVONNE OLSSON,<sup>1</sup> PENELOPE L. NAYUDU,<sup>2</sup> AND ÅKE LUNDWALL<sup>1</sup>

<sup>1</sup>Department of Laboratory Medicine, Lund University, University Hospital MAS, Malmö, Sweden

<sup>2</sup>Department of Reproductive Biology, German Primate Centre, Göttingen, Germany

**ABSTRACT** Human seminal plasma contains high concentrations of prostatic acid phosphatase (PAP), prostate-specific antigen (PSA), beta-microseminoprotein (MSP), semenogelin I (SgI), and semenogelin II (SgII), whereas only PAP and MSP are present in rodents. In order to gain a better understanding of the evolution and function of semen proteins, we have studied ejaculates from the common marmoset (*Callithrix jacchus*)—a New World monkey. Semen samples were analyzed with SDS-PAGE, Western blotting, and isoelectric focusing. Under reducing conditions the dominating protein components appear as heterogeneous material of 55–70 kDa and distinct protein bands of 85, 17, 16, and 15 kDa. The heterogeneous material contains glycosylated material detected by an antiserum recognizing both human SgI and SgII. Southern blotting indicates that the common marmoset has genes for both SgI and SgII. There are several marmoset *MSP* genes, but the strong immunoreactivity against one 15 kDa semen component with pI 7.3 suggests preferential expression of one gene in the prostate. Expression of two other genes cannot be excluded as indicated by weak reaction to isoforms with pI 6.6 and 4.9. Unexpectedly, PSA was not detected by either immunological methods or activity measurements. This is in agreement with results from Southern blotting suggesting that the common marmoset might not have a *PSA* gene. Thus, in this study we have shown that semen coagulum proteins are present in marmoset seminal plasma, but the lack of PSA precludes a similar liquefaction as of human semen. *Mol. Reprod. Dev.* 71: 247–255, 2005. © 2005 Wiley-Liss, Inc.

**Key Words:** male reproductive tract; prostate; seminal vesicle; sperm

## INTRODUCTION

At ejaculation spermatozoa from the epididymis mix with secretion from the accessory sex glands, mainly the seminal vesicles and the prostate, and in some mammals this results in the formation of a semen coagulum (Shivaji et al., 1990). In humans the coagulum

is liquefied within a short time, but in other species, e.g., rodents, it is stabilized by the cross-linking action of a prostate-secreted transglutaminase (Williams-Ashman et al., 1980). The predominant protein components of the human semen coagulum are the seminal vesicle-secreted semenogelin I (SgI) and semenogelin II (SgII) with some contribution also by fibronectin (Lilja et al., 1987). Liquefaction takes place as the result of proteolytic degradation of the coagulum proteins by prostate-secreted proteases, of which prostate specific antigen (PSA) is the most important (Lilja, 1985).

Semen samples from Swedish men have a mean volume of 3.2 ml and contain approximately  $7 \times 10^7$  spermatozoa/ml (Richthoff et al., 2002). The major protein components are, in addition to the already mentioned PSA and proteolytic fragments of SgI and SgII, the prostatic acid phosphatase (PAP), and beta-microseminoprotein (MSP). There is also a prostate-secreted transglutaminase (TGp), but in contrast to the rodent protein that is involved in cross-linking of seminal vesicle-secreted proteins, the function of the human TGp is not known (Grant et al., 1994; Dubbink et al., 1996). Important non-protein components of seminal plasma in man are zinc and fructose. The former is a marker of prostate secretion and has a mean concentration of 2.0 mM in man, and the latter with a mean concentration around 12.7 mM is a marker of seminal vesicle secretion (Elzanaty et al., 2002).

Human SgI and SgII have molecular weights of 50 and 63 kDa, respectively (Lilja et al., 1989; Lilja and Lundwall, 1992). SgII appears in two forms that differ slightly in size due to glycosylation (Malm et al., 1996). Structural studies have shown that SgI and SgII are related and 79% of the primary structure is conserved between them. Furthermore, the major part of the

Grant sponsor: The Swedish Cancer Society (project no. 4564).

\*Correspondence to: Camilla Valtonen-André, Wallenberg Laboratory, University Hospital MAS, S-205 02 Malmö, Sweden.  
E-mail: C.Valtonen-Andre@klkemi.mas.lu.se

Received 7 July 2004; Accepted 9 December 2004

Published online in Wiley InterScience (www.interscience.wiley.com).  
DOI 10.1002/mrd.20257



molecules consists of tandem-repeats of 60 amino acid residues that show varying degree of conservation. As a consequence, antisera raised against either molecule cross-react with multiple epitopes in both molecules (Lilja and Laurell, 1985). Studies of primate DNA have shown that Old World monkeys carry a *SgI* gene that is similar in size to the human orthologue, but that the *SgII* gene is extended by tandem repeats encoding 60 amino acid residues (Ulvsbäck and Lundwall, 1997). Recent studies of hominoid species have shown a reverse pattern, with an extended *SgI* gene and a *SgII* gene that is relatively conserved in size (Jensen-Seaman and Li, 2003; Kingan et al., 2003). There was also a surprising discovery of premature stop codons that give rise to truncated isoforms of both *SgI* and *SgII*. This structural variation of semenogelin molecules between hominoids was suggested to reflect a sexual selection. A similar reasoning was proposed in a study on copulatory plug formation and sexual behaviour that indicates a linkage between promiscuity and formation of a firm semen coagulum (Dixon and Anderson, 2002). Among the New World monkeys, it has been demonstrated that the cotton top tamarin (*Saguinus oedipus*) carries an extended *SgI* but no *SgII* gene (Lundwall, 1998). From the common marmoset a cDNA has been cloned, encompassing an unspliced transcript of *SgI* (Huber et al., 1998). The marmoset has been reported to form a loose coagulum that is similar in consistency to the human one (Dixon and Anderson, 2002). To our knowledge there is no publication on liquefaction of the marmoset seminal coagulum.

Human PSA is a 28.5 kDa glycoprotein that belongs to the glandular kallikrein family—a subfamily of the serine proteases (Lilja, 1985). The closest relatives are the human glandular kallikrein 2 (hK2) and tissue kallikrein, to which PSA displays sequence similarity of 78% and 67%, respectively (Lundwall and Lilja, 1987; Schedlich et al., 1987). These proteins have been intensely studied and measurement of PSA in blood plasma or serum is an important and widely used method for detection and management of prostate cancer. The PSA molecule is secreted as an inactive zymogen that following activation manifests a chymotrypsin-like proteolytic activity (Robert et al., 1997; Malm et al., 2000). The probable physiological activator of PSA in semen is hK2 (Kumar et al., 1997; Lövgren et al., 1997; Takayama et al., 1997). The activity is regulated by different protease inhibitors and also of  $Zn^{+}$  ions that have a strong inhibitory effect on the PSA activity (Malm et al., 2000). Because of their close similarity, antisera and most monoclonal antibodies raised against PSA cross-react with hK2 (Lövgren et al., 1995). Phylogenetic studies show that PSA and hK2 were formed by duplication after the separation of the primates and murine lineages (Olsson and Lundwall, 2002). Furthermore, the duplication must also have happened prior to the separation of Old World monkeys from the lineage leading to humans, as PSA transcripts can be retrieved from prostate tissue of the rhesus monkey (Gauthier et al., 1993).

Human MSP is a 10.7 kDa, non-glycosylated protein that was first isolated from seminal plasma (Johansson et al., 1984; Seidah et al., 1984; Akiyama et al., 1985). The gene of *MSP* gives rise to a transcript that is translated to a polypeptide of 114 amino acid residues, including a signal peptide of 20 residues (Mbikay et al., 1987). The protein has been identified in many different tissues, often in association with mucous secretion, but the function remains unknown (Ulvsbäck et al., 1989; Weiber et al., 1990). The peptide chain has 10 Cys residues that are preserved in different animal species, but the remainder of the primary structure shows high diversity that suggests a rapid evolution: e.g., the rat homologue is only 45% similar to the human protein (Fernlund et al., 1996; Lazure et al., 2001). Studies on the cotton top tamarin surprisingly showed that this New World monkey has at least three *MSP* genes and Southern blotting indicates that the closely related common marmoset has approximately the same number of genes (Mäkinen et al., 1999).

Comparative studies of humans and rodents reveal a remarkably fast evolution of the primary structure of the seminal plasma proteins (Lundwall and Lazure, 1995; Fernlund et al., 1996; Olsson and Lundwall, 2002). Although, very interesting from a phylogenetic point of view, the biological significance is unclear. Most of the research on seminal plasma proteins has been done on non-primates and there are very few studies on primates, except those conducted on the human proteins. Studies of evolutionary more distant primates, such as New World monkeys, could bridge the gap and yield a more comprehensive picture of the evolution of this interesting group of proteins. Therefore, the present study was undertaken in order to characterize components in semen from the common marmoset, a representative New World primate species of the *Callitrichinae* sub-family.

## MATERIALS AND METHODS

### Specimens

Marmoset semen samples were obtained from the colony at the German Primate Centre, Göttingen, Germany. There was a random selection of adult males available in the colony, with ages from 3- to 9-year-old. They had not been subjected to prior experimental studies and their in vivo fertility had not previously been tested. Ejaculates were collected by penile vibrostimulation (Kuederling et al., 2000). Semen volumes were calculated from the weights of the samples, assuming a density of 1 g/ml. The samples were then diluted to 100  $\mu$ l with TALP medium (Gilchrist et al., 1997), and the total number of spermatozoa was calculated from counts of diluted samples in a Neubauer counting chamber (Assistant Glassware Manufacture, Sondheim Germany) using a modification of the standard WHO method for counting human sperm (WHO, 1999). Analysis of seminal plasma was done on semen samples collected from 10 males (1–3 samples each). The average ejaculate volume and total number of spermatozoa were calcu-



lated from 93 samples for which there were 57 values on volume and 89 values on number of spermatozoa. Samples for protein studies were directly frozen in dry ice, but in the case of the stability studies they were first incubated at 37°C for varying periods of time. Prior to experiments, the samples were thawed and diluted in either 25 mM Tris-HCl pH 9.7, 25 mM EDTA, 40 mM DTT, and 4 M urea (stop-buffer) or 20 mM Tris-HCl pH 8.0, 0.15 M NaCl (TBS) and cells were removed by centrifugation at 2000 × g for 5 min. The human semen samples came from volunteer donors attending the Fertility Clinic, University Hospital MAS, Malmö, Sweden. Samples were either allowed to liquefy at room temperature for 1 hr or taken in stop buffer as previously described (Malm et al., 1996). The seminal plasma samples were stored at -20°C.

### Proteins and Antibodies

Human PSA and MSP, kindly provided by Hans Lilja and Per Fernlund, University Hospital MAS, Malmö, Sweden, were purified as described (Lilja and Abrahamsson, 1988; Christensson et al., 1990). Rabbit antisera raised against human PSA, MSP, and a 52 amino acid residue-fragment of SgI have been described earlier (Lilja et al., 1984; Abrahamsson et al., 1988; Weiber et al., 1990). The monoclonal antibodies against human PSA (2E9 and H117) and human SgI (mak1, mak9, and mak17) have also been described elsewhere (Bjartell et al., 1996; Piironen et al., 1998). All antibodies against SgI also show immunoreactivity against SgII. The rabbit antiserum against human PAP was purchased (Dako A/S, Copenhagen, Denmark). Alkaline phosphatase-conjugated goat-antibodies against rabbit IgG and rabbit-antibodies against mouse IgG were from Promega (SDS, Falkenberg, Sweden).

### Analytical Methods

SDS-PAGE was performed in 12% polyacrylamide gels using the Mini PROTEAN II system (Bio-Rad Laboratories AB, Sundbyberg, Sweden) (Laemmli, 1970). Western blotting was done on PVDF membranes (Millipore AB, Sundbyberg, Sweden) after transfer with a semi-dry electroblotting apparatus (Ancos, Hoejby, Denmark) (Towbin et al., 1979). Immunostaining was made with alkaline phosphatase-conjugated secondary antibodies or enhanced chemoluminescence with Vectastain ABC (Vector laboratories, Inc., Burlingame, CA) and the ECL Western Blotting Reagents (Amersham Biosciences, Uppsala, Sweden).

Quenching of the SgI immunoreaction was done by preincubation with human seminal plasma. A sample of 25 µl of unliquefied human seminal plasma in stop buffer was mixed with 50 µl rabbit anti-SgI antiserum in 10 ml of 20 mM Tris-HCl pH 8.0, 0.15 M NaCl with 0.05% Tween 20. After 10 min of incubation at room temperature, the blocked antiserum was used to probe a Western blot.

For deglycosylation experiments, freshly frozen marmoset ejaculate was diluted 1:5 in 150 mM NaCl, 20 mM Tris-HCl pH 7.6, centrifuged at 20,000 × g for 7 min, and

stored at -20°C. Thawed plasma (2 µl) was incubated with 8 U of recombinant *N*-glycosidase F (Roche Diagnostics Scandinavia, Bromma, Sweden) at 37°C for 18 hr, in a final volume of 80 µl buffer (50 mM sodium phosphate pH 7.2 and 12.5 mM EDTA).

Isoelectric focusing was performed on Multiphor II, using ampholyte covering the interval between pH 3.0–10.0 (pharmalyte 3–10 for IEF, Amersham Biosciences), essentially according to (Jeppsson and Berglund, 1972). Press-blot transfer to PVDF membrane was done in 20 mM Tris-HCl pH 7.6 and 150 mM NaCl. Immunodetection was done as for the Western blotting.

PSA activity was measured using a fluorogenic substrate, consisting of the tetrapeptide SerSerTyrTyr conjugated to aminomethylcoumarin (SSYY-AMC) (Niemela et al., 2002). Prior to activity measurements samples were adjusted to a final volume of 198 µl in 50 mM Tris-HCl pH 7.6 containing 0.1 M NaCl and 0.2% BSA—with or without 0.8 mM EGTA. The reactions were initiated by the addition of 2 µl of 20 mM SSYY-AMC in DMSO and the fluorescence was measured in a Victor<sup>2</sup> 1420 multilabel counter (Wallac, Turku, Finland). Final dilution of human and marmoset semen samples were 1:250 and 1:125, respectively. Complexed and free PSA concentrations were measured using the Dual PSA F/T-kit (Wallac, Turku, Finland).

The concentration of zinc in seminal plasma was determined with a colorimetric method and the fructose concentration was measured using a spectrophotometric method essentially as previously described (Wetterauer and Heite, 1978; Makino et al., 1982).

### Southern Blotting

Isolation of DNA from marmoset liver has been described earlier (Ulvbäck and Lundwall, 1997). Aliquots of 15 µg of genomic DNA were digested for 20 hr with 50 U of restriction enzyme in 150 µl of buffer provided with the enzymes (Promega). Thereafter, the material was extracted with phenol, precipitated by ethanol and resolubilized in 20 µl of 10 mM Tris-HCl pH 8.0 and 0.1 mM EDTA. The restricted DNA was separated by electrophoresis in 0.7% agarose gel and transferred to HybondN membrane (Amersham Biosciences) by standard procedures. The probes for hybridization were a cDNA for human PSA, a *Xba*I-*Nco*I fragment encompassing exon 2 of the cotton top tamarin tissue kallikrein gene, and BP1025, containing a *Bam*HI-*Pst*I fragment from the second exon of the human *SgII* gene (Lundwall and Lilja, 1987; Lundwall and Lazure, 1995; Olsson et al., 2000). The probes were labeled with [ $\alpha$ -<sup>32</sup>P]-dCTP to a specific activity exceeding 10<sup>9</sup> dpm/µg, using the Megaprime labeling system (Amersham Biosciences). Prehybridisation and hybridisation were performed at 42°C in ULTRAHYB (Ambion, Inc., Intermedica, Stockholm, Sweden) according to the manufacturer's recommendations. Hybridized membranes were washed several times in 2 × SSPE (1 × SSPE is 0.15 M NaCl, 1 mM EDTA, 10 mM sodium phosphate pH 6.8), 0.1% SDS at room temperature and then in 0.2 × SSPE, 0.5% SDS at 60°C for 30 min. The



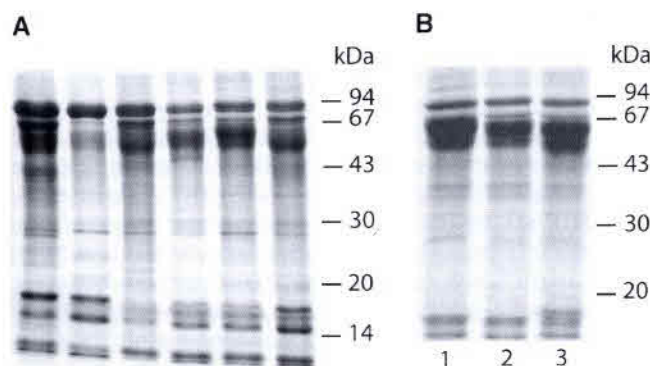
temperature of the stringent wash was increased to 65°C following hybridization with the PSA probe. Autoradiograms were generated using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

## RESULTS

### The Composition of the Marmoset Ejaculate

The seminal plasma was analyzed in ejaculates from 10 animals. The appearance of semen from these males varied greatly with respect to amount of coagulum, volume, and sperm concentration: the recorded median volume was 21  $\mu$ l (range 2–55  $\mu$ l, N=57), and the median number of total ejaculated spermatozoa was  $11 \times 10^6$  (range 0.05–63  $\times 10^6$ , N=89). The material was either a single large mass or smaller clumps of material. It did not spontaneously liquefy, but it was readily dissolved in a moderately denaturing buffer containing urea, indicating that it was not stabilized by transglutaminase-mediated cross-links. The concentrations of zinc and fructose were measured in seminal plasma samples from seven animals. The mean fructose concentration was 17.2 mM (range 13.0–33.0 mM) and the mean  $Zn^{2+}$  concentration was 0.45 mM (range 0.23–1.07 mM).

The protein pattern in reduced samples of marmoset seminal plasma was analyzed by SDS-PAGE (Fig. 1A). Heterogeneous protein components of 55–70 kDa dominated the stained gels, but there were also minor components of 85, 27, 17, 16, and 15 kDa. As can be seen, the relative concentrations of the seminal plasma proteins show significant inter-individual variation. The stability of the protein components was investigated by incubating fresh ejaculates at 37°C for varying periods of time. Analysis by SDS-PAGE shows that the mobility of the dominating components of 55–70 kDa is shifted as to suggest a slight reduction in size, while other molecular species remain relatively unaffected (Fig. 1B). The sample incubated for 24 hr demonstrates a



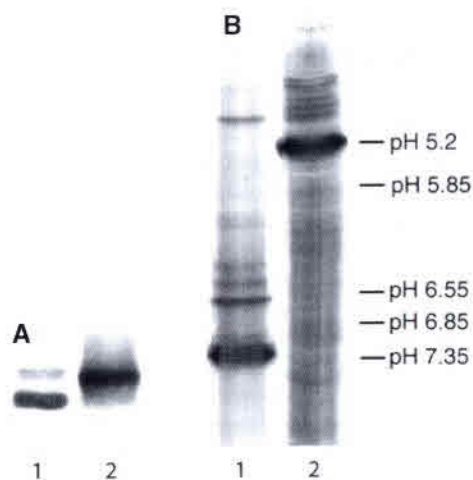
**Fig. 1.** Marmoset seminal plasma proteins. Semen samples (0.4  $\mu$ l) were separated by SDS-PAGE and the gels were stained by Coomassie brilliant blue. Sizes of molecular standards are indicated to the right. **A:** Samples from six males demonstrate protein components of 85, 27, 17, 16, and 15 kDa and heterogeneous material of 55–70 kDa. **B:** Stability of the marmoset seminal plasma proteins demonstrated by incubation at 37°C of three fresh ejaculates from one male, 0 (lane 1), 7 (lane 2), and 24 hr (lane 3), respectively.

reduction of the 55–70 kDa-material by 5–10 kDa, to stable end products.

### Prostate-Secreted Proteins

Western blotting, using a polyclonal antiserum against human MSP, showed weak but specific immunoreactivity with a 15 kDa component in marmoset seminal plasma (Fig. 2A). The apparent size is slightly less than that of human MSP, which migrates as a 16 kDa protein. Isoelectric focusing demonstrated a single molecular species with pI of 5.3 in the human sample, whereas in the marmoset sample there is one major band of MSP immunoreactivity with pI around 7.3 and also at least two weak but distinct bands with pI of approximately 6.6 and 4.9 (Fig. 2B).

Marmoset seminal plasma was also investigated with respect to the prostate-secreted PAP and PSA, but the available antisera showed no immunostaining in Western blotting. Further attempts to demonstrate PSA was done by a variety of methods. Several monoclonal antibodies and a polyclonal antiserum raised against human PSA, but also recognizing conserved epitopes in human hK2, were used for immunological detection of PSA. However, neither by Western blotting nor by the sensitive Dual PSA F/T assay could any PSA immunoreactivity be demonstrated. Seminal plasma was also investigated with respect to amidolytic activity against the peptide substrate SSSY-AMC that shows specificity for PSA. As can be seen, the activity in marmoset seminal plasma is negligible compared to the activity in human seminal plasma or that exerted by purified human PSA (Fig. 3). The experiment was made in the presence of EGTA to chelate endogenous zinc that might interfere with the PSA activity measurements.



**Fig. 2.** Immunological detection of MSP. **A:** Western blot with 0.6  $\mu$ l marmoset seminal plasma (lane 1) and 1.5  $\mu$ g purified human MSP (lane 2). **B:** Isoelectric focusing of 2  $\mu$ l marmoset seminal plasma (lane 1) and 2  $\mu$ l human seminal plasma (lane 2). Immunological staining is visualised after transfer of protein to PVDF membrane, showing one major and two minor MSP isoforms in the marmoset sample and one single human MSP.



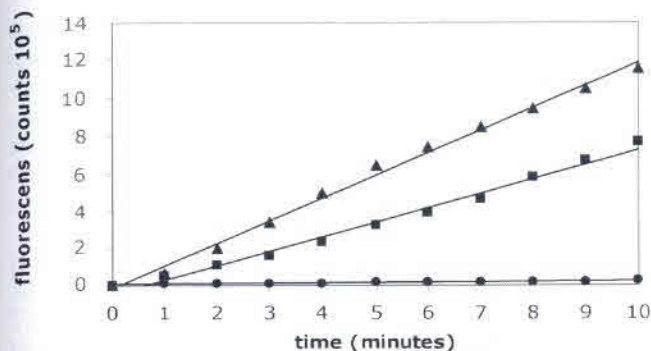


Fig. 3. PSA activity measurements. The fluorogenic substrate SSYY-AMC was incubated with 1.6  $\mu$ l marmoset seminal plasma (circle), 0.8  $\mu$ l human seminal plasma (squares), and 1.25  $\mu$ g/ml purified human PSA (triangles).

The failure to detect PSA in marmoset seminal plasma prompted an investigation of glandular kallikrein genes in genomic DNA. A Southern blot was probed by DNA-fragments encompassing either exon 2 of the cotton top tamarin tissue kallikrein gene or a cDNA encoding human PSA. Both probes stained two *Eco*RI-fragments and two *Xba*I-fragments of equal sizes but with different intensity, as to suggest that two related genes are present in the marmoset genome (Fig. 4). The >15 kb *Eco*RI and the 4.4 kb *Xba*I fragments are presumably from the tissue kallikrein gene, while the 13 kb *Eco*RI and the 10 kb *Xba*I fragments represent a gene that is more closely related to the PSA gene.

#### Seminal Vesicle-Secreted Proteins

The polyclonal antiserum, raised against a 50 amino acid peptide-fragment of human SgI, yielded a weak immunostaining of an approximately 65 kDa-component in marmoset seminal plasma. The monoclonal antibodies used gave no reaction. In order to exclude that the weak immunoreactivity was due to unspecific binding of antibodies to highly charged semenogelin molecules, the specificity of the signal was tested by a blocking experiment. As can be seen, the immunostaining is almost completely abolished by preincubation of the antiserum with unliquefied human seminal plasma (Fig. 5).

The Western blotting showed that the heterogeneous material of 55–70 kDa in marmoset ejaculates is related to SgI, but, in contrast to the human analogue, it appears to be relatively stable. Because of this, experiments were conducted on marmoset seminal plasma samples in order to explain the difference in stability. In a first experiment, divalent cations, like  $Zn^{2+}$ , were chelated by EGTA. Following incubation at 37°C, the protein pattern was analyzed with SDS-PAGE. The samples containing EGTA did not significantly differ from those incubated without EGTA, indicating that the increased stability of marmoset Sg is independent of  $Zn^{2+}$ . In a second experiment the resistance of marmoset Sg to degradation by proteases was studied by incubating with human PSA. To assure that the enzyme was functional in marmoset seminal

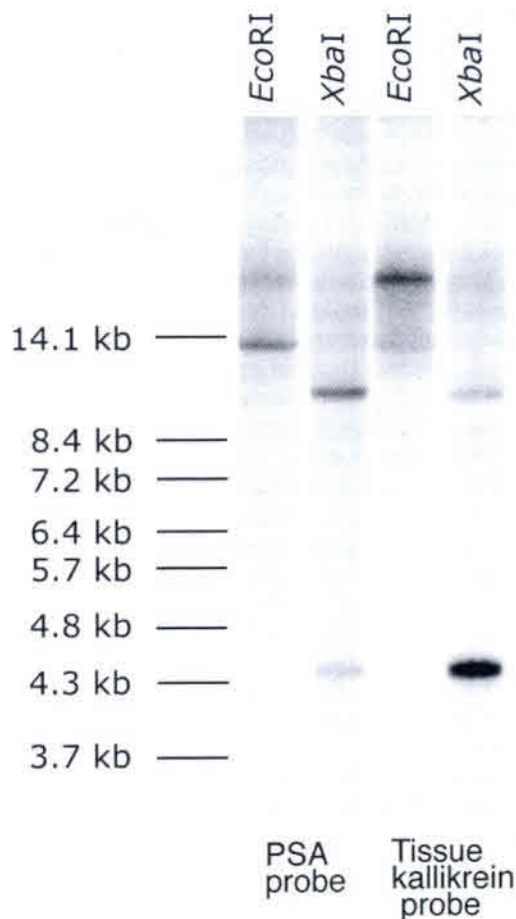


Fig. 4. Southern blotting with kallikrein probes. The autoradiogram is shown after hybridization of marmoset DNA with the PSA probe (two left lanes) and the tissue kallikrein probe (two right lanes). Restriction enzymes are shown on top and size markers are indicated to the left. Two *Eco*RI-fragments and two *Xba*I-fragments of equal sizes but with different intensity are visualized.

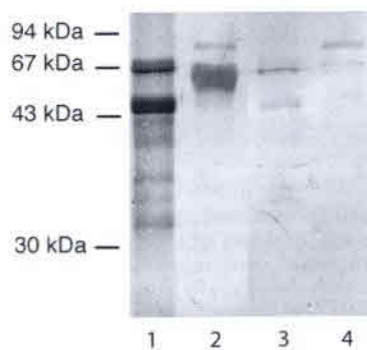
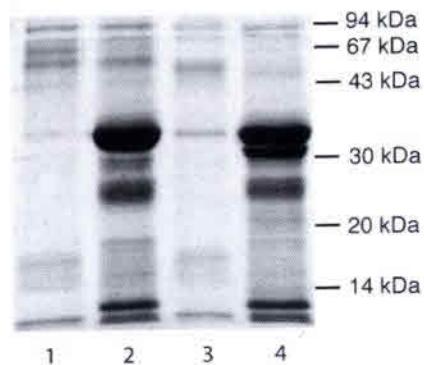


Fig. 5. Demonstration of semenogelin immunoreactivity. Western blot with 0.2  $\mu$ l unliquefied human seminal plasma (lanes 1 and 3) and 0.3  $\mu$ l marmoset seminal plasma (lanes 2 and 4) was developed with a polyclonal antiserum against human SgI. The antiserum used for samples in lanes 3 and 4 was preincubated with unliquefied human seminal plasma. Molecular size markers are indicated to the right.

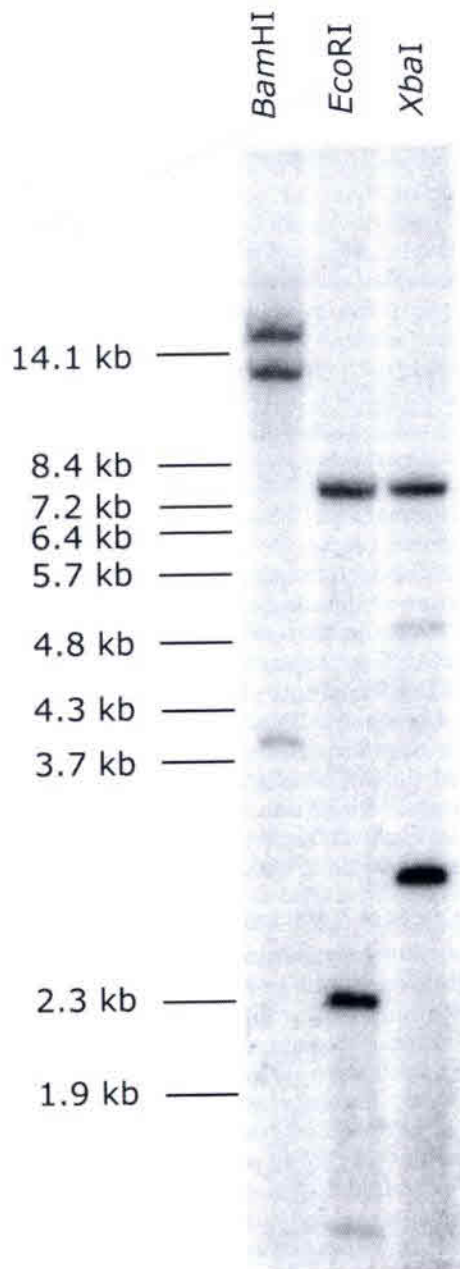


plasma, the activity of exogenously added PSA was first monitored using the fluorogenic substrate SSYY-AMC. The activity of purified human PSA was as high in marmoset seminal plasma as in buffer, suggesting that endogenous protease inhibitors did not significantly affect human PSA activity. Incubation with human PSA at 0.7 mg/ml for 3 hr, showed a slight degradation of the 55–70 kDa material, resembling the pattern observed after incubation of seminal plasma at 37°C for 24 hr. Thus, it appears as if the central part of the marmoset Sg molecule is resistant to degradation by both endogenous proteases as well as exogenously added PSA. An analysis of the primary structure, deduced from the published cDNA (Huber et al., 1998) suggests that marmoset SgI has a molecular size of approximately 48 kDa and carries eight sites for potential *N*-linked glycosylation. Thus, a high degree of glycosylation could explain the protease resistance. Treatment of the marmoset seminal plasma with *N*-glycosidase reduced the heterogeneity and apparent molecular mass of the 55–70 kDa-material to approximately 50 kDa, indicating deglycosylation. Furthermore, deglycosylation also rendered the material susceptible to degradation by PSA, which shows that the protease resistance was glycosylation-dependent (Fig. 6)

Western blotting showed one homogeneous band of Sg immunoreactivity, indicative of a single molecular species in marmoset semen and not two as in humans. Because of the discrepancy, the number of Sg genes was examined by Southern blotting. Using a probe recognizing both SgI and SgII, two strongly hybridising fragments were seen in DNA samples cleaved with *Bam*HI, *Eco*RI and *Xba*I (Fig. 7). This suggests that the genome of the common marmoset contains two semenogelin genes.



**Fig. 6.** Proteolytic sensitivity after deglycosylation. Marmoset seminal plasma was incubated with *N*-glycosidase for 18 hr at 37°C and subjected to digestion with human PSA. Samples equivalent to 0.08  $\mu$ l seminal plasma were analyzed by SDS-PAGE. The gel stained with Coomassie brilliant blue is shown with size markers indicated to the left; seminal plasma without additions (lane 1), seminal plasma digested with PSA for 3 hr (lane 2), deglycosylated seminal plasma (lane 3), deglycosylated seminal plasma digested with PSA for 3 hr (lane 4). After deglycosylation, the heterogeneity and apparent molecular mass of the 55–70 kDa-material was reduced to relatively homogeneous material of approximately 50 kDa. The deglycosylated material was also susceptible to degradation by PSA. The prominent bands in lanes 2 and 4 are from the exogenously added PSA.



**Fig. 7.** Southern blotting with semenogelin probe. The autoradiogram is shown after hybridization with the BP1025, recognizing exon two of both the human *SgI* and *SgII* gene. Restriction enzymes are shown on top and size markers are indicated to the left. Two strongly hybridising fragments can be seen after cleavage with *Bam*HI, *Eco*RI, and *Xba*I suggesting two semenogelin genes.

## DISCUSSION

Semen samples from the common marmoset were obtained by penile vibrostimulation, a method that is thought to give a balance of seminal plasma components most similar to a natural ejaculate. It has been shown that this method yields three to four times more spermatozoa than that obtained by rectal probe electro-ejaculation (Schneiders et al., 2004). In our study,



the median values for semen volume and the number of spermatozoa are a bit lower compared to those previously reported for penile vibrostimulation (Kuederling et al., 2000). The reason for this is not clear, but inclusion in the study of very small samples and preanalytical factors like diet or general state of health could be of importance.

Compared to human samples, the  $Zn^{2+}$  concentration is low. Even the sample with the highest level was still 30% lower in  $Zn^{2+}$  than an average human ejaculate. This might be regarded as an indication of low contribution by the prostate to marmoset ejaculates, but it could also reflect a relatively lower concentration of  $Zn^{2+}$  in secretions from the marmoset prostate gland.

The seminal plasma proteins are dominated by heterogeneous material of 55–70 kDa. Much of the heterogeneity appears to be related to carbohydrates as it is substantially decreased by *N*-glycosidase digestion. The material shows relatively weak immunoreactivity with an antiserum that detects repeated epitopes in human SgI. Although the antiserum was raised against a proteolytic fragment of human SgI, it also recognizes human SgII. This suggests that the repeated epitope very likely is conserved also in the marmoset, as the duplication that yielded SgI and SgII took place prior to the separation of New World monkeys from the lineage leading to humans. Results from this investigation, in combination with the previous cloning of cDNA from a seminal vesicle library, clearly demonstrates that SgI is a major protein component in marmoset seminal plasma. In contrast to the cotton-top tamarin, in which the *SgII* gene is deleted, the common marmoset might have both a *SgI* and a *SgII* gene (Ulvsbäck and Lundwall, 1997). In the present study Southern blotting clearly shows that the common marmosets do have two *Sg* genes. However, it was not possible to decide whether both genes were functional and expressed in the seminal vesicles. Such studies have to await the arrival of more specific tools.

Marmoset seminal plasma is composed of both fluid and coagulum fractions, and it appears not to undergo a liquefaction process. At the molecular level this is supported by the fact that the presumed coagulum proteins of 55–70 kDa are only slightly affected by spontaneous proteolysis, even after incubation at 37°C for 24 hr. The absence of PSA, which plays an important role in liquefaction of human semen by degrading SgI and SgII, is not the critical difference, as addition of human PSA only slightly affects the coagulum proteins in marmoset semen. Instead, resistance to liquefaction is probably due to the extensive glycosylation, leading to protection of the major part of SgI against proteolytic degradation. In the light of these results it is possible to speculate whether the expression of the *PSA* gene was lost in the marmoset prostate due to a loss of biological significance as a consequence of the protease resistant *Sg* molecules.

DNA hybridization and sequence analysis have shown that the cotton-top tamarin has a tissue kallikrein gene and one or possibly two genes related to PSA/

hK2 (Olsson et al., 2000). The results from the Southern blotting in this report lead to similar conclusions about the common marmoset, and it appears quite clear that the marmoset only carry two kallikrein genes—one tissue kallikrein gene and one PSA/hK2-related gene. Because of the high sequence similarity between PSA and hK2, around 80% in man, the 13 kb *EcoRI* and the 10 kb *XbaI* fragments stained by the PSA-probe could represent a *PSA* gene, a *hK2* gene or a precursor of both. Most likely, it does not represent a *PSA* gene, given the absence of PSA in marmoset seminal plasma: the absence of the *PSA* gene in the very closely related cotton top tamarin also supports this hypothesis (Olsson et al., 2004).

PAP is a relatively preserved molecule with more than 80% sequence conservation between man and mouse. Therefore, it would be expected that an antiserum raised against human PAP has a high probability of cross-reacting with the marmoset protein. Consequently, the failure to detect this protein would indicate that the marmoset prostate does indeed not secrete PAP. The functional significance of this is to be determined.

The common marmoset and the cotton-top tamarin, the two New World monkeys so far studied, carry several *MSP* genes. Other mammals, including man, have one gene only. Analysis of marmoset DNA by Southern blotting suggests that the most likely number of *MSP* genes in this species is four (Mäkinen et al., 1999). Three *MSP* molecules from the cotton top tamarin have been analyzed with respect to primary structure and they are all of equal size with a molecular mass of 10.7 kDa. Western blotting of marmoset seminal plasma detects material with *MSP* immunoreactivity that migrates on SDS-PAGE with an apparent molecular size of 15 kDa. The mobility of the marmoset *MSP* differs from the human protein that migrates as a 16-kDa protein, even though it has a mass of 10.8 kDa. The human and tamarin proteins are of similar molecular size and it is therefore probable that also the marmoset *MSP* has a molecular mass of around 10.7 kDa. The difference in mobility on SDS-PAGE is probably a reflection of difference in SDS-binding rather than size. The immunoreactive material on the Western blot could represent one or several components. The marmoset, like the tamarin, could be expected to have several equally sized *MSP* molecules. The isoelectric focusing shows that one *MSP* molecule is dominating in marmoset seminal plasma, but that two additional molecular species are present at lower concentration. In humans, *MSP* is expressed in many different tissues. This could also be the case in the marmoset, but there is also a possibility for tissue specific expression of the different molecular forms in this species. Such additional complexity could perhaps aid in the understanding of the biological function of *MSP*.

In conclusion, this study has provided the first basic information regarding several key seminal plasma proteins and their genes in the common marmoset monkey. We have shown that this New World primate species most likely has two *Sg* genes, of which at least



one is expressed and secreted into seminal plasma. The finding of both the Sg and MSP homologues, imply some evolutionary value of these proteins with respect to reproduction. PAP and PSA, however, have not been found in seminal fluid. The absence of PSA together with the lack of liquefaction in marmoset semen is especially interesting, since the proteolytic activity of this protease is responsible for the degradation of the gel proteins and release of progressively motile spermatozoa in humans. However, the biological and evolutionary significance of these findings is not yet clear and further studies of these proteins with emphasis on gene-structure, evolution and function in both humans and other closely related primate species will be required.

#### ACKNOWLEDGMENTS

Margareta Persson is acknowledged for excellent technical assistance and Nicole Umland for skilled technical assistance in the collection and evaluation of the samples.

#### REFERENCES

- Abrahamsson PA, Lilja H, Falkmer S, Wadstrom LB. 1988. Immunohistochemical distribution of the three predominant secretory proteins in the parenchyma of hyperplastic and neoplastic prostate glands. *Prostate* 12:39-46.
- Akiyama K, Yoshioka Y, Schmid K, Offner GD, Troxler RF, Tsuda R, Hara M. 1985. The amino acid sequence of human beta-microseminoprotein. *Biochim Biophys Acta* 829:288-294.
- Bjartell A, Malm J, Moller C, Gunnarsson M, Lundwall Å, Lilja H. 1996. Distribution and tissue expression of semenogelin I and II in man as demonstrated by in situ hybridization and immunocytochemistry. *J Androl* 17:17-26.
- Christensson A, Laurell CB, Lilja H. 1990. Enzymatic activity of prostate-specific antigen and its reactions with extracellular serine proteinase inhibitors. *Eur J Biochem* 194:755-763.
- Dixon AL, Anderson MJ. 2002. Sexual selection, seminal coagulation and copulatory plug formation in primates. *Folia Primatol (Basel)* 73:63-69.
- Dubbink HJ, Verkaik NS, Faber PW, Trapman J, Schroder FH, Romijn JC. 1996. Tissue specific and androgen-regulated expression of human prostate-specific transglutaminase. *Biochem J* 315:901-908.
- Elzanaty S, Richthoff J, Malm J, Giwercman A. 2002. The impact of epididymal and accessory sex gland function on sperm motility. *Hum Reprod* 17:2904-2911.
- Fernlund P, Granberg LB, Larsson I. 1996. Cloning of beta-microseminoprotein of the rat: A rapidly evolving mucosal surface protein. *Arch Biochem Biophys* 334:73-82.
- Gauthier ER, Chapdelaine P, Tremblay RR, Dube JY. 1993. Characterization of rhesus monkey prostate specific antigen cDNA. *Biochim Biophys Acta* 1174:207-210.
- Gilchrist RB, Nayudu PL, Hodges JK. 1997. Maturation, fertilization, and development of marmoset monkey oocytes in vitro. *Biol Reprod* 56:238-246.
- Grant FJ, Taylor DA, Sheppard PO, Mathewes SL, Lint W, Vanaja E, Bishop PD, O'Hara PJ. 1994. Molecular cloning and characterization of a novel transglutaminase cDNA from a human prostate cDNA library. *Biochem Biophys Res Commun* 203:1117-1123.
- Huber AE, Fraser H, del Rio-Garcia J, Kreil G, Smyth DG. 1998. Molecular cloning in the marmoset shows that semenogelin is not the precursor of the TRH-like peptide pGlu-Glu-Pro amide. *Biochim Biophys Acta* 1387:143-152.
- Jensen-Seaman MI, Li WH. 2003. Evolution of the hominoid semenogelin genes, the major proteins of ejaculated semen. *J Mol Evol* 57:261-270.
- Jeppsson JO, Berglund S. 1972. Thin-layer isoelectric focusing for haemoglobin screening and its application to haemoglobin Malmö. *Clin Chim Acta* 40:153-158.
- Johansson J, Sheth A, Cederlund E, Jornvall H. 1984. Analysis of an inhibin preparation reveals apparent identity between a peptide with inhibin-like activity and a sperm-coating antigen. *FEBS Lett* 176:21-26.
- Kingan SB, Tatar M, Rand DM. 2003. Reduced polymorphism in the chimpanzee semen coagulating protein, semenogelin I. *J Mol Evol* 57:159-169.
- Kuederling I, Schneiders A, Sonksen J, Nayudu PL, Hodges JK. 2000. Non-invasive collection of ejaculates from the common marmoset (*Callithrix jacchus*) using penile vibrostimulation. *Am J Primatol* 52:149-154.
- Kumar A, Mikolajczyk SD, Goel AS, Millar LS, Saedi MS. 1997. Expression of pro form of prostate-specific antigen by mammalian cells and its conversion to mature, active form by human kallikrein 2. *Cancer Res* 57:3111-3114.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Lazure C, Villemure M, Gauthier D, Naude RJ, Mbikay M. 2001. Characterization of ostrich (*Struthio camelus*) beta-microseminoprotein (MSP): Identification of homologous sequences in EST databases and analysis of their evolution during speciation. *Protein Sci* 10:2207-2218.
- Lilja H. 1985. A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. *J Clin Invest* 76:1899-1903.
- Lilja H, Abrahamsson PA. 1988. Three predominant proteins secreted by the human prostate gland. *Prostate* 12:29-38.
- Lilja H, Laurell C-B. 1985. The predominant protein in human seminal coagulate. *Scand J Clin Lab Invest* 45:635-641.
- Lilja H, Lundwall Å. 1992. Molecular cloning of epididymal and seminal vesicular transcripts encoding a semenogelin-related protein. *Proc Natl Acad Sci USA* 89:4559-4563.
- Lilja H, Laurell CB, Jeppsson JO. 1984. Characterization of the predominant basic protein in human seminal plasma, one cleavage product of the major seminal vesicle protein. *Scand J Clin Lab Invest* 44:439-446.
- Lilja H, Oldbring J, Rannevik G, Laurell CB. 1987. Seminal vesicle-secreted proteins and their reactions during gelation and liquefaction of human semen. *J Clin Invest* 80:281-285.
- Lilja H, Abrahamsson PA, Lundwall Å. 1989. Semenogelin, the predominant protein in human semen. Primary structure and identification of closely related proteins in the male accessory sex glands and on the spermatozoa. *J Biol Chem* 264:1894-1900.
- Lundwall Å. 1998. The cotton-top tamarin carries an extended semenogelin I gene but no semenogelin II gene. *Eur J Biochem* 255:45-51.
- Lundwall Å, Lazure C. 1995. A novel gene family encoding proteins with highly differing structure because of a rapidly evolving exon. *FEBS Lett* 374:53-56.
- Lundwall Å, Lilja H. 1987. Molecular cloning of human prostate specific antigen cDNA. *FEBS Lett* 214:317-322.
- Lövgren J, Piironen T, Overmo C, Dowell B, Karp M, Pettersson K, Lilja H, Lundwall Å. 1995. Production of recombinant PSA and HK2 and analysis of their immunologic cross-reactivity. *Biochem Biophys Res Commun* 213:888-895.
- Lövgren J, Rajakoski K, Karp M, Lilja H, Lundwall Å. 1997. Activation of the zymogen form of prostate-specific antigen by human glandular kallikrein 2. *Biochem Biophys Res Commun* 238:549-555.
- Makino T, Saito M, Horiguchi D, Kina K. 1982. A highly sensitive colorimetric determination of serum zinc using water-soluble pyridylazo dye. *Clin Chim Acta* 120:127-135.
- Malm J, Hellman J, Magnusson H, Laurell CB, Lilja H. 1996. Isolation and characterization of the major gel proteins in human semen, semenogelin I and semenogelin II. *Eur J Biochem* 238:48-53.
- Malm J, Hellman J, Hogg P, Lilja H. 2000. Enzymatic action of prostate-specific antigen (PSA or hK3): Substrate specificity and regulation by Zn(2+), a tight-binding inhibitor. *Prostate* 45:132-139.
- Mbikay M, Nolet S, Fournier S, Benjannet S, Chapdelaine P, Paradis G, Dube JY, Tremblay R, Lazure C, Seidah NG, Cretien M. 1987. Molecular cloning and sequence of the cDNA for a 94-amino-acid seminal plasma protein secreted by the human prostate. *DNA* 6:23-29.



- Mäkinen M, Valtonen-André C, Lundwall Å. 1999. New world, but not Old world, monkeys carry several genes encoding beta- microseminoprotein. *Eur J Biochem* 264:407-414.
- Niemela P, Lövgren J, Karp M, Lilja H, Pettersson K. 2002. Sensitive and specific enzymatic assay for the determination of precursor forms of prostate-specific antigen after an activation step. *Clin Chem* 48:1257-1264.
- Olsson AY, Lundwall Å. 2002. Organization and evolution of the glandular kallikrein locus in *Mus musculus*. *Biochem Biophys Res Commun* 299:305-311.
- Olsson AY, Persson AM, Valtonen-André C, Lundwall Å. 2000. Glandular kallikreins of the cotton-top tamarin: Molecular cloning of the gene encoding the tissue kallikrein. *DNA Cell Biol* 19:721-727.
- Olsson AY, Valtonen-André C, Lilja H, Lundwall Å. 2004. The evolution of the glandular kallikrein locus: Identification of orthologs and pseudogenes in the cotton top tamarin. *Gene* 343:347-355.
- Piironen T, Villoutreix BO, Becker C, Hollingsworth K, Vihinen M, Bridon D, Qiu X, Rapp J, Dowell B, Lövgren T, Pettersson K, Lilja H. 1998. Determination and analysis of antigenic epitopes of prostate specific antigen (PSA) and human glandular kallikrein 2 (hK2) using synthetic peptides and computer modeling. *Protein Sci* 7:259-269.
- Richthoff J, Rylander L, Hagmar L, Malm J, Giwercman A. 2002. Higher sperm counts in Southern Sweden compared with Denmark. *Hum Reprod* 17:2468-2473.
- Robert M, Gibbs BF, Jacobson E, Gagnon C. 1997. Characterization of prostate-specific antigen proteolytic activity on its major physiological substrate, the sperm motility inhibitor precursor/semenogelin I. *Biochemistry* 36:3811-3819.
- Schedlich LJ, Bennetts BH, Morris BJ. 1987. Primary structure of a human glandular kallikrein gene. *DNA* 6:429-437.
- Schneiders A, Sonksen J, Hodges JK. 2004. Penile vibratory stimulation in the marmoset monkey: A practical alternative to electroejaculation, yielding ejaculates of enhanced quality. *J Med Primatol* 33:98-104.
- Seidah NG, Arbatti NJ, Rochemont J, Sheth AR, Chretien M. 1984. Complete amino acid sequence of human seminal plasma beta-inhibin. Prediction of post Gln-Arg cleavage as a maturation site. *FEBS Lett* 175:349-355.
- Shivaji S, Scheit K-H, Bhargava PM. 1990. *Proteins of seminal plasma*. New York: John Wiley & Sons, Inc.
- Takayama TK, Fujikawa K, Davie EW. 1997. Characterization of the precursor of prostate-specific antigen. Activation by trypsin and by human glandular kallikrein. *J Biol Chem* 272:21582-21588.
- Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 76:4350-4354.
- Ulvsbäck M, Lundwall Å. 1997. Cloning of the semenogelin II gene of the rhesus monkey. Duplications of 360 bp extend the coding region in man, rhesus monkey and baboon. *Eur J Biochem* 245: 25-31.
- Ulvsbäck M, Lindstrom C, Weiber H, Abrahamsson PA, Lilja H, Lundwall Å. 1989. Molecular cloning of a small prostate protein, known as beta-microseminoprotein, PSP94 or beta-inhibin, and demonstration of transcripts in non-genital tissues. *Biochem Biophys Res Commun* 164:1310-1315.
- Weiber H, Andersson C, Murne A, Rannevik G, Lindstrom C, Lilja H, Fernlund P. 1990. Beta microseminoprotein is not a prostate-specific protein. Its identification in mucous glands and secretions. *Am J Pathol* 137:593-603.
- Wetterauer U, Heite HJ. 1978. Carnitine in seminal fluid as parameter for the epididymal function. *Andrologia* 10:203-210.
- WHO. 1999. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge: Cambridge University Press.
- Williams-Ashman HG, Bell RE, Wilson J, Hawkins M, Grayhack J, Zunamon A, Weinstein NK. 1980. Transglutaminase in mammalian reproductive tissues and fluids: Relation to polyamine metabolism and semen coagulation. *Adv Enz Regul* 18:239-258.