Purification, characterization, and molecular processing of the precursor of a sperm motility inhibitor present in human seminal plasma.

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Abstract

Human seminal plasma contains a protein factor that can inhibit the motility of both demembranated-reactivated and intact spermatozoa. This factor, named seminal plasma motility inhibitor (SPMI), was originally isolated from seminal plasma and shown to originate exclusively from the seminal vesicles, where its specific activity is 5- to 10-fold higher than it is in seminal plasma. The present study aimed at investigating the mechanism responsible for this difference in activity. Analysis of semen after ejaculation allowed to demonstrate that this difference in SPMI specific activity is due to the presence of a predominant 52 kDa SPMI precursor form in seminal vesicle fluid which is rapidly degraded after ejaculation by prostatic proteases. In addition, SPMI precursor was found to be associated with semen coagulum proteins and abnormal processing of the precursor in semen was associated with poor sperm motility.

A novel method was developed to purify SPMI precursor from seminal vesicle fluid and semen coagulum. Prostate-specific antigen (PSA) hydrolyzed SPMI precursor in a manner reminiscent of its processing in whole semen. Biochemical analysis of the precursor protein and its hydrolysis products provided evidence that SPMI precursor is identical to semenogelin, the main structural protein of semen coagulum. The purified precursor inhibited the motility of intact spermatozoa in a reversible and dose-dependent manner.

Finally, the characterization of SPMI molecular processing by PSA was addressed. The results directly demonstrate for the first time the restricted chymotrypsin-like specificity of PSA on its major physiological substrate. The sites of hydrolysis by PSA were identified along the precursor molecule and specific domains within the SPMI precursor responsible for

biological activity and reactivity with various antibodies were mapped.

Overall, these results shed light on the proteolytic processing of SPMI precursor occurring after ejaculation, and the associated change in SPMI activity on spermatozoa. The present findings provide evidence, for the first time, that a specific protein appears responsible for the observed low sperm motility in freshly ejaculated semen, and that its processing by PSA parallels the progressive increase in sperm motility observed during semen liquefaction.

Résumé

Le plasma séminal humain contient une protéine qui a la capacité d'inhiber la mobilité des spermatozoïdes démembranés-réactivés ou intacts. Ce facteur, nommé "seminal plasma motility inhibitor" (SPMI), fut initialement isolé à partir du plasma séminal humain et provient uniquement des sécrétions des vésicules séminales où son activité spécifique est de 5 à 10 fois plus élevée que celle retrouvé dans le plasma séminal. La présente étude visait donc à élucider le mécanisme expliquant cette différence d'activité. L'analyse du semen après éjaculation a permis de conclure que cette différence d'activité est due au fait que dans le fluide des vésicules séminales, le SPMI est présent sous la forme d'un précurseur de 52 kDa qui est rapidement dégradé éjaculation par des protéases prostatiques. De plus, le précurseur du SPMI fut retrouvé parmi les composantes coagulées du semen et l'absence de dégradation normale du précurseur dans le sperme a pu être associé à une faible mobilité des spermatozoïdes.

Un nouveau procédé de purification du précurseur de SPMI a été développé. Nous avons démontré que l'antigène spécifique de la prostate (PSA) peut hydrolyser le précurseur du SPMI de la même façon que dans le sperme. L'analyse biochimique du précurseur et de certains produits d'hydrolyse a permis de conclure que le précurseur du SPMI est identique à la "semenogelin", principale composante du coagulum séminal. Le précurseur du SPMI purifié inhibe la mobilité des spermatozoïdes intacts en fonction de la concentration de SPMI et de facon réversible.

Pour terminer, une analyse détaillée du processus de transformation moléculaire du précurseur du SPMI par le PSA fut entreprise. Les résultats ont permis de démontrer que l'activité et

la spécificité de type chymotrypsine du PSA sur son substrat physiologique prédominant est inhabituelle. Les différents sites d'hydrolyse par PSA furent identifiés, permettant ainsi de localiser la région du présurseur du SPMI responsable de l'activité biologique ainsi que les épitopes reconnus par différents anticorps.

Globalement, ces résultats permettent de mieux comprendre le processus de transformation moléculaire du précurseur du SPMI après éjaculation. Les résultats permettent de démontrer, pour la première fois que le précurseur du SPMI semble responsable de la faible mobilité des spermatozoïdes dans le sperme fraîchement éjaculé et que sa transformation moléculaire par le PSA se fait en parallèle avec l'augmentation progressive de mobilité des spermatozoïdes se produisant durant la liquéfaction du sperme.

List of abbreviations

AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
API-MS	atmospheric pressure ionization mass spectrometry
ATP	adenosine triphosphate
BCIP	bromo-chloro-indolphosphate
BSP	bovine seminal protein
CAPS	cyclohexylaminopropane sulfonic acid
cAMP	3'-5' cyclic adenosine monophosphate
DFP	diisopropyl fluorophosphate
cDNA	complementary deoxyribonucleic acid
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
FPLC	fast protein liquid chromatography
g	gravity constant
HBS	HEPES buffered saline
HEPES	N-2-hydroxyethyl piperazine N-2'-ethanesulphonic acid
hGK-1	human glandular kallirein-1
HSS	HEPES saline solution
IGF	insulin-like growth factor
IgG	gamma-immunoglobulin

kDa	kiloDalton
Mg.ATP	magnesium adenosine triphosphate
NBT	nitroblue tetrazolium chloride
PF	prostate fluid
NPGB	para-nitrophenylguanidobenzoate
pI	isoelectric point
PSA	prostate-specific antigen
PMSF	phenylmethylsulphonyl fluoride
RNA	ribonucleic acid
RP-HPLC	reverse-phase high performance liquid chromatography
SD	standard deviation
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SPMI	seminal plasma motility inhibitor
STI	soybean trypsin inhibitor
SVF	seminal vesicle fluid
SVSA	seminal vesicle-specific antigen
TBS	TRIS buffered saline
TFA	trifluoroacetic acid
TLCK	N-α-tosyl-L-lysine-chloromethylketone
TPCK	tosyl-L-phenylalanine-chloromethylketone
TRIS	tris-(hydroxymethyl) aminomethane

Preface

Guidelines from the thesis office

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis. If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the

doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.**

The above described option was chosen in preparing the present thesis. The first chapter is an introduction containing a review of the literature and presenting the objectives and rationale of the present study. Chapters II and III consist of reprints of published papers from the *International Journal of Andrology* and *Human Reproduction.* Written permission has been obtained from the respective publishers to reproduce integrally these published articles. Chapters IV and V are composed of two manuscripts to be published. Collectively, these chapters form the logical progression of the present investigation. Chapters II to VI are preceded by short introductory texts that provide a link between them. The references found at the end of the thesis are valid for Chapters I and VI. The references for Chapters II to V are found at the end of the respective chapter.

Apart from the following exceptions, all of the experimental work, data collection, and analysis described in the present work were performed by M. Robert under the supervision of Dr. C. Gagnon.

- In chapter IV, the amino acid analyses were performed by Susan James of the endocrine laboratory, Royal Victoria Hospital, Montréal, Québec.

- In chapter IV and V, the N-terminal sequence analyses were performed at the Sheldon Biotechnology Center and the mass spectrometry analysis was performed by Bernard Gibbs at the Biotechnology Research Institute of the National Research Council of Canada, Montréal, Québec.
- Elka Jacobson, a summer research student under the immediate supervision of the author contributed to some of the results related to the effect of various protease inhibitors on PSA activity found in chapter V.

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I dedicate this thesis to my wife, Kazuyo, who has made it all worthwhile, for her constant support, encouragement, patience, confidence and love, to my mother, Thérèse, who always guided me through the sometimes difficult choices and to my father, Maurice, who instilled in me this sense of curiosity.

Chapter I

Introduction

Introduction

1.1 Overview

Reproduction, or the begetting of descendants and the associated recombination and transmission of genes is, together with feeding, the main basic preoccupation that all living organisms share. It is no surprise then, that during development of this function throughout evolution, nature has "given birth" to an astonishing array of different processes and mechanisms to achieve this ultimate goal. This variety probably reflects efforts to optimize chances of fertilization success and, at the same time, provide a uniqueness to most species. From the most basic process of binary fission in unicellular organisms to the extremely sophisticated process of mammalian reproduction, the variation on this theme never ceases to puzzle and fascinate the human mind. It appears overwhelming that, in mammals, such a complex mechanism starting from gametogenesis and leading to fertilization and zygote development manages to be so efficient. However, in spite of the high success rate and survival of species through millions of years of evolution, the reproductive function of all living species is not immune to malfunctions. For humans, failure to reproduce has always been a great source of concern. History's earliest records make reference to infertility and all cultures possess rituals, prayers or ceremonies to induce fertility.

It was the Dutch microscopist Antoni van Leeuwenhoek (1678) who first reported the presence of spermatozoa in semen. He was also first to recognize that their presence in semen and their ability to move and survive sufficiently long in the female genital tract were the essential prerequisites to a man's fertility potential. It is now reported that about 15% of couples in North America face infertility problems (Hargreave, 1984). Traditionally, for

various historical and socio-cultural reasons, failure to conceive was associated almost exclusively to failures occurring in the female's reproductive system. For this reason, the study of andrology (the male counterpart to gynecology) was considerably neglected. However, more recently, male factors were found to be directly responsible for 30% of cases in infertile couples, and to play a role in an additional 20% of cases (Amelar et al., 1977). These changes in perception led to the development of andrology as a sub-speciality of urology (or obstetrics and gynecology) and reproductive medicine. However, most processes concerning the endocrinology of the male reproductive system, the complex process of spermatogenesis, the role of the accessory glands and the various steps of the sperm journey from the male reproductive tract to the site of fertilization remain poorly characterized. It is thus a formidable challenge, where most mechanisms remain to be uncovered, that many scientists have undertaken with great enthusiasm. The field has thus expanded exponentially in the recent years especially with the development of novel and powerful molecular approaches to probe the function of the various components. The male germ cells are a splendid model of cellular differentiation while at the same time they retain the genetic pluripotentiality that will allow the growth of a new complete organism after the fusion with the egg.

The present project was undertaken in an environment where the emphasis in research is at the level of the control of sperm motility and sperm function. Most of the investigations aim at highlighting specific mechanisms and factors, whether beneficial or detrimental, involved in the regulation of sperm motility and sperm functions. The applied objective is to elucidate some of the mechanisms underlying male factor infertility and to develop avenues for new treatments.

Obviously, in a world where almost every life form, from the most basic bacteria to humans, expresses some form of sexuality, female reproductive function bears an importance equal to that of males for successful reproduction. However, for sake of brevity and clarity, the present review focusses on male reproductive function. Moreover, due to the extraordinary variety of reproductive processes between the different living organisms and species we will, in the present study, focus our attention on mammalian species. The present introduction proposes a general overview of the basic male reproductive function with particular emphasis on sperm motility and functions as well as on the secretions of the male accessory glands and the biochemistry of semen since these have particular relevance to the experimental component of the present work.

1.2 Male reproductive system

Among all physiological systems known, it is within the reproductive system, and more specifically among the so-called male accessory sex glands, that the widest variations between species exist (Mann and Lutwak-Mann, 1981; Coffey, 1994), both anatomically and biochemically. Little is known about the need for such diversity. However, this wide variety is suggested to reflect adaptation of species to various environmental exposures, to differences in reproductive behavior that contribute to minimize exposure to pathogens, and to avoid inter-species breeding and injuries to the reproductive system (Coffey, 1994).

A diagram of the human male reproductive system is presented in Figure 1. The basic organ of the male reproductive tract is the testis and its main secretory products are the spermatozoa and testosterone. This male hormone is mainly responsible for the male reproductive tract differentiation and the induction of spermatogenesis. Anatomically, the testis is mainly

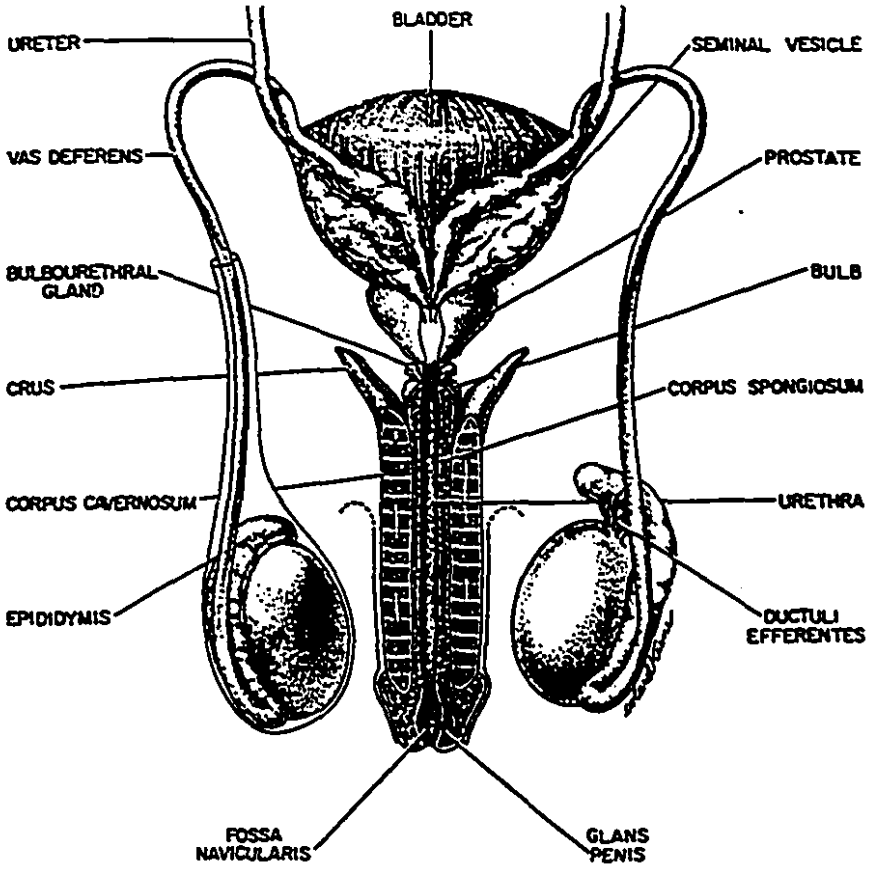

Figure 1. Posterior view of the human male reproductive system. From Dym. M. (1977) The male reproductive system. In: *Histology*. Weiss L., Greep R.O. editors. McGraw-Hill Book Co. New York

constituted of seminiferous tubules at the base of which the epithelium, and more specifically, the Sertoli cells are actively involved in the coordination of spermatogenesis. The Leydig cells, on the other hand, are found in the interstitial space and are responsible for the synthesis of testosterone (Setchell and Brooks, 1994). Spermatozoa produced in the testis travel to the epididymis, where sperm maturation and storage take place. The epididymis is a long convoluted tubular structure (about 6-7 meters in man) secreting various proteins and low molecular weight compounds that interact with spermatozoa in specific regions of the epididymis (Hoskins and Vijayaraghavan, 1990). It is during epididymal transit that spermatozoa acquire the capacity to move forward and gain their fertilizing ability (Eddy and O'Brien, 1994). The vas deferens is a tubule connecting the epididymis to the ejaculatory duct. Its main function is its contractile activity which propels spermatozoa toward the distal regions of the reproductive tract. However, it also demonstrates limited secretory activity making a minor contribution to semen. The distal end of the vas deferens is terminated by an anatomical enlargement, the ampulla, located near the site of entrance within the prostate, at the level of the so-called ejaculatory duct.

Humans, rats and boars all have large elongated seminal vesicles whereas these glands are absent in dogs and cats (Mann and Lutwak-Mann, 1981). Carpi, who first described these structures in 1521, thought that they functioned merely as storage for semen, hence the misnomer. However, these structures are now known to actively secrete numerous semen components (Aumüller and Riva, 1992). The seminal vesicles rest in the connective tissue on the posterior side of the bladder, adjacent to the rectum. Recent evidence suggests that the seminal vesicles, along with the ampulla of the vas deferens and the ejaculatory duct can be considered functionally as a single unit, called the ampullo-vesiculo-ductal complex (Riva et

al., 1989).

In contrast to the seminal vesicles, the prostate gland is common to all mammalian species even though there also exist large variations amongst different species. In humans, the prostate surrounds the proximal urethra immediately below the bladder base. Whereas the rat prostate is macroscopically divided into three distinct lobes, each having a specialized function, the human and dog prostates have a much more uniform morphological appearance. While the role of the prostatic secretions remains poorly characterized, the cellular components of the gland have been extensively studied since this organ is particularly prone to abnormal growth and carcinoma in aged men (Aumüller and Seitz, 1990). Within the prostate gland, the vas deferens and the seminal vesicles merge to form the ejaculatory ducts. The latter terminate at the level of the prostatic urethra, a site known as the verumontanum (Coffey, 1994). Finally, along the urethra are found minor glands, the paired pea-size bulbourethral or Cowper's gland found below the prostate and the Littre's gland lining the penile urethra. The accessory glands of the male reproductive tract are actively involved in *de novo* synthesis and secretion of numerous specific substances that do not simply originate from serum by transudation. However, the exact biological functions of all these accessory glands and their secretory products are poorly understood (Coffey, 1994).

Ejaculation is triggered by sequential contractions of multiple pelvic muscles. This results in the propulsion of epididymal spermatozoa along the efferent ducts and urethra with concomitant emptying of the various gland contents. There is thus both a cellular component (spermatozoa) and a soluble component (seminal plasma) in semen. All these components are not emitted simultaneously, but rather sequentially (Tauber et al., 1975, 1976). The first fraction of the ejaculate contains mainly prostatic secretions. The second fraction contains

spermatozoa and epididymal components while the contents of the seminal vesicles are emitted in the last fraction (Lindholmer, 1973).

1.3 Androgen action on reproductive organs.

Male sexual development and maturation of reproductive organs are closely linked to androgen action. The male hormone testosterone and its metabolite dihydrotestosterone (DHT) are responsible for the onset of spermatogenesis at puberty (Steinberger et al., 1973), the development and maturation of the various glands along the reproductive system, semen production and male secondary sexual characteristics (Mann and Lutwak-Mann, 1981). In the accessory sex glands, circulating testosterone enters the cell by simple or facilitated diffusion, and is metabolized by the enzyme 5α-reductase which converts it into the more potent DHT (Aümuller and Seitz, 1990). DHT is then transported to the nucleus where its effects are mediated through binding to the intracellular androgen receptor (Zhou et al., 1994). Both testosterone and DHT can bind to the receptor. The hormone/receptor complex then binds to specific DNA elements, modifying the topology of the chromatin, and resulting in increased transcription of specific genes. Despite enormous advances in the study of the steroid family of nuclear receptors at the molecular level, relatively few genes whose expression is directly regulated by androgens have been characterized. Some of these include the mouse mammary tumor virus promoter (Cato et al., 1987), the rat SVS IV and V proteins (Higgins and Hemingway, 1991), a mouse vas deferens protein (Fabre et al., 1994), and an elastase-like protease in the guinea pig seminal vesicle (Harvey et al, 1995). In addition, little is known about the specific DNA sequences with which the hormone/androgen receptor complex interacts. However, an androgen response element was identified in the promoter

of the prostate-specific antigen gene (Riegman et al, 1991) and evidence for binding of the androgen receptor to this element was recently provided (Luke and Coffey, 1994).

1.4 The spermatozoon

The basic functions of the male gamete are to carry a haploid copy of the male's genome in close proximity to the female gamete within the female genital tract, to penetrate the egg and to achieve fertilization. As we will see below, the spermatozoon possesses remarkably specialized features that allow it to perform its function successfully. These characteristics paradoxically reflect extensive morphological differentiation while, at the same time, the genetic material of the cell remains developmentally totipotent (Eddy and O'Brien, 1994). Spermatogenesis has its origins within the epithelium of the seminiferous tubules of the testis where, following a complex series of mitotic divisions, spermatogonial stem cells undergo two successive meiotic divisions, to produce the spermatids. The latter, following extensive morphological changes are in turn released into the lumen of the seminiferous tubules as spermatozoa. The whole differentiation process takes approximately 60 to 80 days in man (Heller and Clermont, 1964). As spermatozoa leave the testis, they are fully differentiated but do not yet possess the ability to move effectively (Eddy and O'Brien, 1994). Spermatozoa are then transported to the epididymis where maturation and storage take place. It is during the epididymal maturation process that spermatozoa acquire the capacity for progressive motility, even though they remain immotile within the epididymis until ejaculation (Eddy and O'Brien, 1994). It is also during epididymal transit that spermatozoa acquire the ability to fertilize the egg (Yanagimachi, 1994).

1.4.1 Morphology

A mammalian spermatozoon is composed of two different parts, a head and a flagellum, which are joined at the neck, as shown in Figure 2. The head consists primarily of a nucleus, the acrosome and a limited amount of cytoplasm and cytoskeleton. The extended tail of spermatozoa, or flagellum, is made up of the axoneme, mitochondria, outer dense fibers, fibrous sheath, and cytoplasm. While all mammalian spermatozoa share these basic features, some differences exist between species in the shape and size of the head and in the length and relative size of the components of the tail (Yanagimachi, 1994).

The plasma membrane surrounds the whole surface of the spermatozoa. This membrane is shaped according to the standard lipid bilayer fluid mosaic model in which various proteins are embedded. The main component phospholipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, phosphatidylinositol, lyso-phosphatidylcholine and lyso-phosphatidylethanolamine together with smaller amounts of neutral lipids and glycolipids (Parks and Hammerstedt, 1985; Mack et al., 1987; Agrawal et al., 1988). Interestingly, the plasma membrane has been found to contain regional domains that differ in composition (Holt, 1984; Wolf and Voglmayr, 1984). This probably reflects the separate functions performed by different parts of the cell (Eddy and O'Brien, 1994). However, the mechanism responsible for the production and maintenance of those domains has not been elucidated. Extensive remodeling of the plasma membrane occurs during epididymal transit (Eddy et al., 1991). These changes affect the composition of both the membrane lipids and proteins, and likely modify membrane fluidity (Yanagimachi, 1994). A good example is that of cholesterol which is actively synthesized and transferred to the plasma membrane during epididymal maturation and appears to participate in plasma membrane

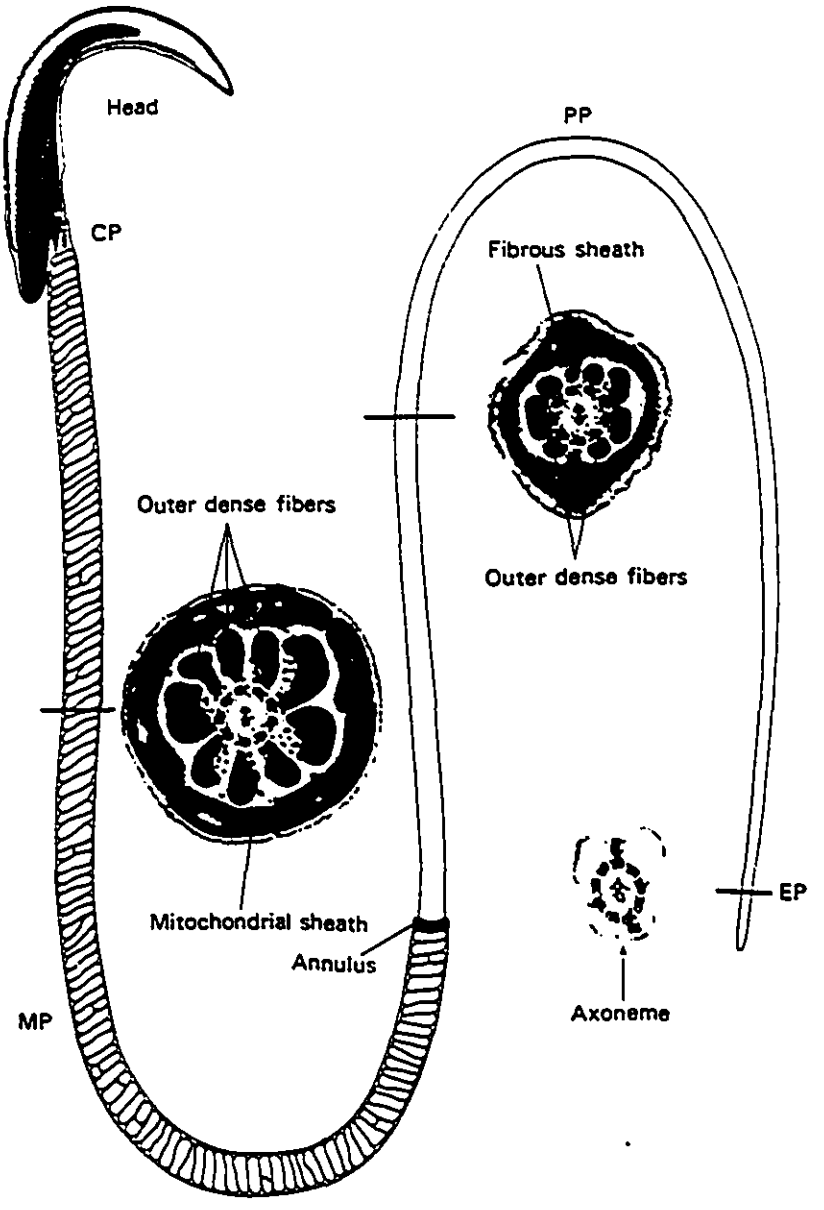

Figure 2. Diagram of a rat spermatozoon. The four regions of the tail are indicated, i.e., the connecting piece (CP) joining the head to the tail, the middle piece (MP) showing the transversely oriented mitochondria, the principal piece (PP), and the end piece (EP). Electron microscope photographs along the tail give the ultrastructure of the regions as seen in cross sections. These show the axoneme (microtubular doublets and singlets), the outer dense fibres, the fibrous sheath with the longitudinal columns and ribs, and mitochondria. The annulus is also indicated at the junction of the middle and principal piece. Modified from Oko and Clermont (1990).

stabilization (Seki et al., 1992). Numerous sperm surface proteins are lost during epididymal maturation, while others are added to the membrane of spermatozoa as integral membrane proteins or simply adsorbed on the cell surface (Eddy and O'Brien, 1994). Some of these proteins may serve as membrane stabilizing factors to prevent a premature acrosome reaction (Reynolds et al., 1989) while others are believed to be involved in sperm-egg interaction at fertilization (Moore et al, 1987; O'Rand et al, 1988). Alterations in sperm-surface composition also occur at ejaculation (Eddy and O'Brien, 1994). All these various changes appear to be necessary for the spermatozoon to acquire the ability to fertilize.

1.4.1.1 Sperm head

One of the specific features of the sperm head is the acrosome, a structure lying immediately anterior to the tip of the sperm head, beneath the plasma membrane (Figure 3). Developmentally, this structure originates from the Golgi apparatus of spermatids (Eddy and O'Brien, 1994). The outer acrosomal membrane directly underlies the plasma membrane of the anterior sperm head while the inner acrosomal membrane overlies the anterior part of the outer nuclear membrane (Eddy and O'Brien, 1994). These membranes define the acrosome which can be most simply symbolized as a sac containing a wide array of potent hydrolytic enzymes that allow the sperm to penetrate the egg's outer vestments before achieving fertilization (Zaneveld and De Jonge, 1991). Its best characterized components are proacrosin, a powerful serine protease and hyaluronidase (Goldberg, 1977; Muller-Esterl and Fritz, 1981). Several other proteases, including phospholipases, glycosidases and esterases also have been detected in the acrosome (Yanagimachi, 1994). Conversion of the inactive zymogen proacrosin to the active enzyme acrosin occurs during the acrosome reaction

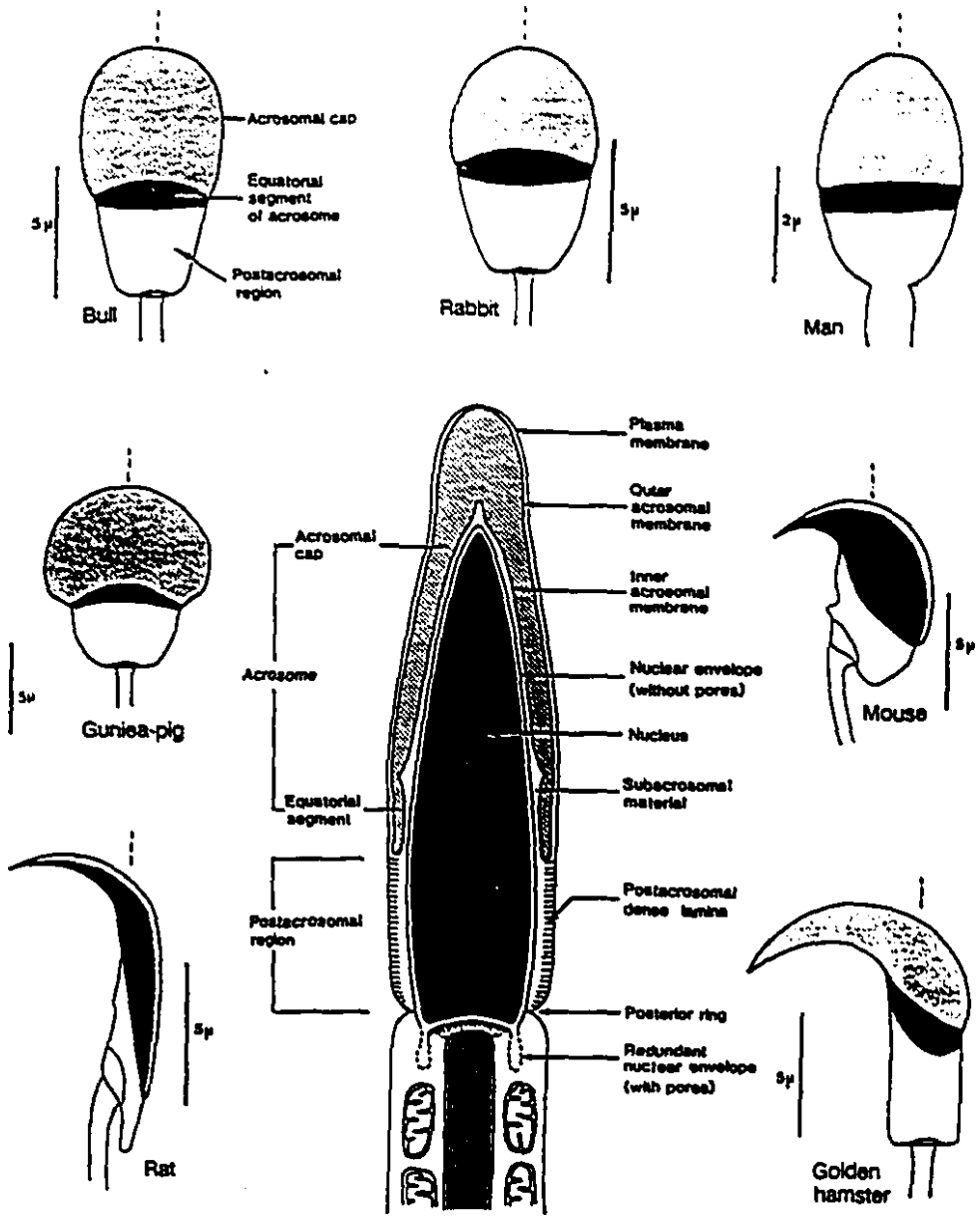

Figure 3. Diagrams illustrating the relative size and topographical relationship between the acrosomal cap and the equatorial segment of the spermatozoa of seven different species. Although shapes and relative size of these two regions of the acrosome vary from species to species, their basic structures are the same. The central figure is a schematic representation of the sagittal section (—) through the head. Modified from Yanagimachi (1994).

(Nuzzo et al, 1990). Acrosin is believed to be the major player in the capacity of spermatozoa to penetrate the egg surface. However, it was recently reported that spermatozoa from mice with a targeted disruption of the acrosin gene can still penetrate the oocyte zona pellucida and effect fertilization (Baba et al., 1994). This finding suggests that acrosin may be dispensable and that alternative enzymes or factors are also very important for fertilization.

The sperm nucleus carries only a single copy of each chromosome. During spermatogenesis, the sperm chromatin is remodeled and becomes highly condensed, allowing the sperm nucleus to occupy a much smaller volume than in somatic cells and assume the relatively narrow and elongated shape typical of the mammalian sperm head (Yasuzumi, 1974). This tight compaction is in part made possible by the replacement of histones, the nuclear proteins present in somatic cells, with the small and very basic sperm specific protamines (Balhorn, 1989; Meistrich, 1989). The protamine bound DNA is coiled into concentric circles that are attached to the nuclear matrix and the nuclear annulus (Ward and Coffey, 1991; Hud et al., 1993; Ward, 1993). This highly ccndensed protamine-DNA complex is stabilized by disulfide bridges between protamines (Kvist, 1980; Yanagimachi, 1994). In its supercondensed state, the sperm chromatin is maintained in a genetically inactive state (Poccia, 1986). The sperm head additionally contains cytoskeleton components (perinuclear theca) thought to define the shape of the sperm head (Olson et al., 1983) but relatively little cytoplasm (Yanagimachi, 1994).

1.4.1.2 The flagellum

Four different segments can be identified along the mammalian sperm flagellum (Figure 2): the connecting piece, the middle piece, the principal piece, and the end piece (Eddy and

O'Brien, 1994). Extending over the full length of the flagellum is the axoneme, a remarkable structure responsible for generating movement (Figure 4). The axoneme is composed of a so-called "9+2" microtubule complex in which a central pair of microtubules is surrounded by nine microtubule doublets, each composed of A and B microtubules or subfibers (Fawcett and Porter, 1954). The microtubules themselves are composed of both α and ß-tubulin isoforms (Hecht et al., 1984). The A microtubule consists of 13 protofilaments, whereas the "C-shaped" B microtubule contains only 11 protofilaments and is attached to the A microtubule (Tilney et al., 1973). Two dynein arms protrude from the A microtubule toward the B microtubule of the adjacent doublet. These projecting arms contain the ATPase activity that is responsible for the generation of movement through the sliding of microtubules upon ATP hydrolysis (Satir, 1979). Additionally, structures known as radial spokes are connected to each outer doublet at the level of the A microtubule and project toward the central pair of microtubules (Bryan and Wilson, 1971). Nexin links are also present in some species, linking adjacent outer doublets by flexible extensions. Finally, besides these major components, the axoneme is composed of numerous other proteins (Luck, 1984).

Additional features of the flagellum of mammalian spermatozoa include the outer dense fibers network and the fibrous sheath whose functions are not clearly understood (Eddy and O'Brien, 1994). Surrounding the outer dense fiber in the middle piece of the sperm tail is an end to end helical arrangement of mitochondria (Phillips, 1977). These organelles are the site of sperm oxidative metabolism where the ATP required for sperm motility is generated (Ford and Rees, 1990).

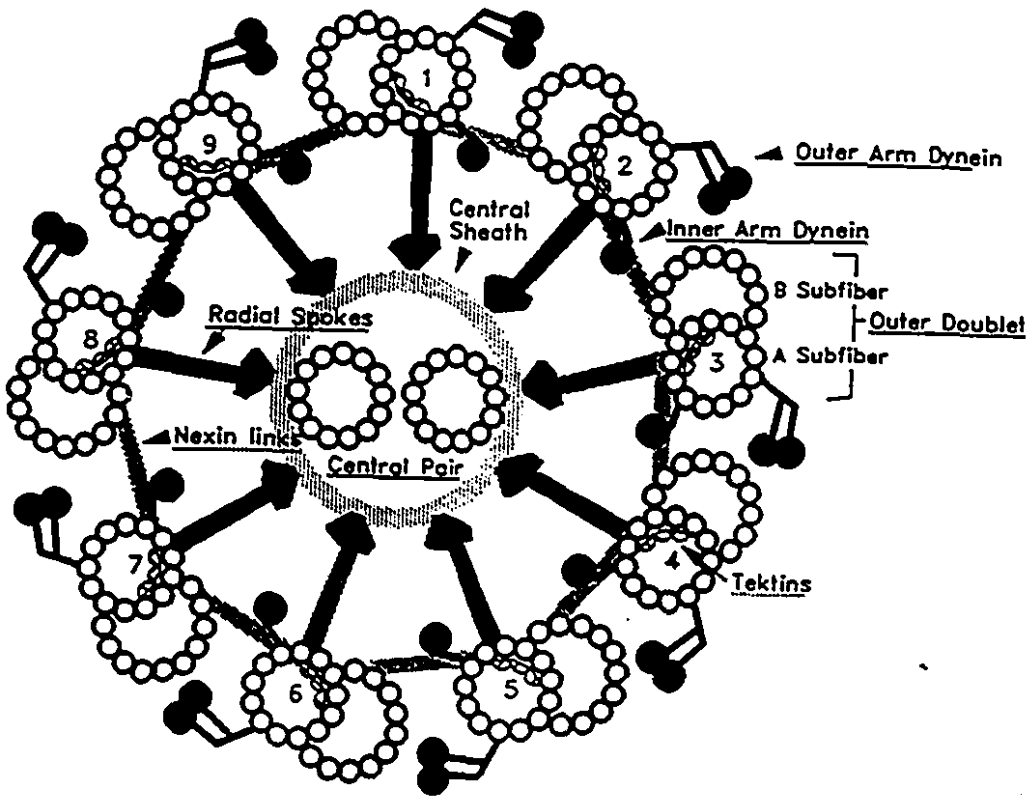

Figure 4. Structure of the sperm axoneme. The diagram shows the main known components, i.e., the microtubule doublets, the dynein arms, the radial spokes and the nexin links. Modified from Tash (1990).

1.4.2 Sperm functions

In order to achieve their ultimate goal, spermatozoa must undertake, following ejaculation, a long and perilous journey through the female reproductive tract up to the Fallopian tubule. Of the tens of millions of spermatozoa deposited in the female tract at ejaculation, only few will reach the site of fertilization. The single most important factor that will allow spermatozoa to reach the vicinity of the egg is motility.

1.4.2.1 Motility: Mechanism and energetics

Sperm motility is by far the most energy demanding process in spermatozoa. The stiffness of the flagellum and the viscosity of the fluid in which spermatozoa are bathed are two interacting factors that must be overcome (Mann and Lutwak-Mann, 1981). The mechanism enabling spermatozoa to develop flagellar motion is linked to the transformation of the chemical energy stored in the ATP pool into mechanical energy (Ford and Rees, 1990). This transformation is mediated by the proteins of the axoneme and more specifically by the action of the dynein arms which contain an ATPase activity (Satir, 1979; Gibbons, 1981). Flagellar movement is initiated when sliding between adjacent outer microtubule doublets occurs through a mechanism reminiscent of the actin-myosin translocation occurring during muscle contraction (Mann and Lutwak-Mann, 1981). The dynein arms form bridges to the adjacent pair of microtubules. Upon ATP binding this attachment is released, and ATP is hydrolyzed causing a translational movement of the arms along the axoneme and translocation (Gibbons and Gibbons, 1974). This attachment-detachment-translocation cycle triggers the sliding of adjacent microtubules against each other resulting in a bending of the flagellum and wave propagation (Gibbons, 1981). The ATP necessary to sustain motility is generated primarily

by the catabolism of different substrates, most notably by glycolysis of fructose and glucose, and oxidative phosphorylation (Ford and Rees, 1990).

Gagnon et al. (1982, 1986) demonstrated the importance of protein carboxymethylase activities in spermatozoa for the maintenance of motility and a role for proteases in the regulation of sperm motility has also been suggested (de Lamirande and Gagnon, 1983; de Lamirande et al., 1986).

1.4.2.2 Capacitation

Of the multiple spermatozoa that reach the vicinity of the oocyte, only those that have undergone a process called capacitation can successfully achieve fertilization. The phenomenon of capacitation was originally discovered following unsuccessful attempts to fertilize eggs *in vitro*, using ejaculated or epididymal spermatozoa. On the other hand, spermatozoa that had resided in the oviduct or uterus could fertilize (Chang, 1951, 1955; Austin, 1951, 1967). This ill-defined process is best described as a series of functional changes in spermatozoa, occurring in the female reproductive tract, that give spermatozoa the competency to undergo the acrosome reaction and achieve fertilization (Yanagimachi, 1994). The heterogeneity of sperm populations has been a major hurdle to the study of capacitation. Traditionally, the capacity of spermatozoa to undergo the acrosome reaction (see below) *in vitro* (triggered by substances such as the calcium ionophore A23187, lyso-phosphatidylcholine or isolated zona pellucida) has been used as an indicator of capacitation. However, it is possible that special conditions may induce the acrosome reaction spontaneously through a pathway that bypasses capacitation. Moreover, spermatozoa which have been capacitated may somehow be prevented to undergo the acrosome reaction. In such

cases, failure of the acrosome reaction does not necessarily imply that spermatozoa were not capacitated (Yanagimachi, 1994). The exact molecular mechanisms responsible for capacitation remain largely unknown, but appear to involve changes in the properties of the sperm plasma membrane (Clegg, 1983), in intracellular ionic composition (Hyne et al., 1985), increases in metabolism and motility parameters (Boell, 1985), and loss of seminal plasma proteins attached to the sperm membrane (Vernon et al., 1985). Some decapacitating factors may be bound to the sperm surface and released only at the proper moment. Potential candidates for this activity were found in different species and showed to inhibit the fertilizing ability of spermatozoa (Bedford and Chang, 1962; Williams et al., 1967; Fraser et al., 1990).

1.4.2.3. Hyperactivation

The phenomenon of sperm hyperactivation refers to a specific type of motility where spermatozoa start to move frantically (Yanagimachi, 1994). Sperm hyperactivation occurs concomitantly with capacitation while the latter is a prerequisite for the acrosome reaction *in vivo* (Burkman, 1990). The motility of hyperactivated spermatozoa is characterized by high velocity, low forward progression and whiplash-like movement of the flagellum (Katz and Yanagimachi, 1980; Burkman, 1990). Generally, there is a good correlation between sperm hyperactivation and sperm ability to penetrate the zona pellucida (Yanagimachi, 1994). Hyperactivation is believed to facilitate the release of spermatozoa from the folds of the oviductal epithelium (Demott and Suarez, 1992), swimming of spermatozoa through the viscous fluid of the oviduct (Suarez et al., 1991; Suarez and Dai, 1992) and penetration through the zona pellucida and other egg vestments by generating strong propulsive thrust (Katz and Yanagimachi, 1981; Suarez et al., 1991). Hyperactivation is believed to be induced

by activation of calcium channels, resulting in transient calcium influx and initiation of the cAMP-protein kinase signalling cascade (Suarez et al., 1993; Yanagimachi, 1994)

1.4.2.4 Acrosome reaction

Once capacitated, spermatozoa can bind to the zona pellucida proteins. This binding induces a cascade of reactions in spermatozoa involving calcium influx and activation of sperm phospholipases that ultimately result in the acrosome reaction (Harrisson and Roldan, 1990; Kopf and Gerton, 1991; Yanagimachi, 1994). The mechanism of the acrosome reaction, which is an exocytotic event, involves a fusion reaction as well as the vesiculation and shedding of the outer acrosomal membrane and the sperm plasma membrane covering the proximal part of the head, down to the equatorial segment (Yanagimachi, 1994). This reaction results in the release of the various acrosomal hydrolytic enzymes which, in turn, allow the spermatozoon to penetrate the vestments surrounding the egg. Human seminal plasma can prevent spermatozoa from undergoing the acrosome reaction (Cross, 1993). The inhibitory factor has recently been identified as cholesterol (Cross, 1996). *In vivo*, the zona pellucida, and progesterone are believed to induce the acrosome reaction (Florman and Storey, 1982; Osman et al., 1989). The zona-induced acrosome reaction is usually species specific but exceptions have been reported (Lee et al., 1987; Uto et al., 1988).

1.4.2.5 Fertilization

After successfully penetrating the zona pellucida, the spermatozoon rapidly crosses the perivitelline space, and reaches the egg plasma membrane. The inner acrosomal membrane then fuses with it (Yanagimachi, 1994). Following internalization of the spermatozoon, the

dormant egg gets activated, resulting in the completion of the second meiosis (Yanagimachi, 1994). During that time, the male nucleus decondenses and transforms into a pronucleus (Zirkin et al., 1989). Finally both pronuclei meet at the center of the egg, their nuclear membranes disintegrate and the diploid cell is now ready to undergo its first division (Yanagimachi, 1994). This stage marks the end of the fertilization process and the beginning of embryo development.

1.5 Biochemistry of seminal plasma

1.5.1 Overview

As described above, seminal plasma forms at ejaculation when the fluids of the various glands of the reproductive tract are mixed. Apart from its obvious role as a hydrodynamic medium for transporting spermatozoa, the physiological function of seminal plasma during the reproductive process has traditionally been underestimated and neglected and thus remains poorly characterized (Coffey, 1994). This is explained in part by the fact that, in various species, successful fertilization has been possible with the use of epididymal spermatozoa, suggesting that seminal plasma is not an absolute requirement for fertility *in vitro* (Eliasson and Johnsen, 1986). However, this does not rule out important functions *in vivo*. There are also various reports demonstrating the importance of the secretions from the accessory glands for fertility. In some species, surgical removal of certain accessory sex glands does not significantly affect fertility (Eliasson and Johnsen, 1986). On the other hand, in the domestic mouse, the seminal vesicles appear to play a major role in fertility (Pang et al., 1979; Peitz and Olds-Clarke, 1986; Peitz, 1988). In rats, ablation of the seminal vesicles rendered males completely infertile (Cukierski et al., 1991). Moreover, while the removal of either the ampullary gland or the ventral prostate in hamsters did not affect fertilization rate, the premature death of embryos suggested an impairment in reproductive efficiency (Chow & O, 1988). These findings suggest that, under special circumstances, fertilization is possible using epididymal spermatozoa and that the factors contributed by seminal plasma may be dispensable *in vitro*. However, it also implies that components of seminal plasma contributed by the accessory glands are very important to achieve fertilization and may be involved in

supporting early embryo development (Chow et al., 1994). Moreover, taking into account the wide variation in accessory glands between species, it would be inappropriate to infer that observations made in one species are necessarily applicable to another (Mann and Lutwak-Mann, 1981). The controversy concerning the physiological relevance of seminal plasma was well summarized by Mann and Lutwak-Mann (1981); " In conclusion, although many unanswered questions remain as to how the various chemical constituents of seminal plasma figure in reproduction, to deny to this fluid any physiological significance whatsoever is neither sensible nor justified.".

Several functions for seminal plasma have been described or hypothesized: modulation of sperm metabolism (Mann and Lutwak-Mann, 1981; Inskeep et al., 1985), immunosuppressive activity to protect spermatozoa from immune cells of the female reproductive tract (James and Hargreave, 1984), stimulation of female reproductive tract smooth muscle contraction (Clavert et al., 1990), protection of spermatozoa against reactive oxygen species (Iwasaki and Gagnon, 1992) and lipid peroxidation (Eliasson and Johnsen, 1986) and the modulation of sperm function and motility (Aumüller et al., 1990; Iwamoto et al, 1990).

In humans, the secretions originating from the prostate contribute around 15 to 25% of the total ejaculate volume, while those of the seminal vesicles account for as much as 60 to 70% (Eliasson and Johnsen, 1986; Aümuller and Riva, 1992; Coffey 1994). The contributions of the vas deferens, ampulla, Cowper's glands and Littre's glands represent less than 10% of the volume (Polakoski et al, 1976). Paradoxically, spermatozoa account for less than 1% of the semen volume (Coffey, 1994).

Seminal plasma is formed by the sequential emission of the various fluids of the male reproductive tract (Tauber et al., 1975, 1976). The ejaculate can thus be split into different

fractions for analysis. Using this approach in humans, the fluids from the prostate and the sperm-rich epididymal fluid were found to be ejaculated first, followed in the late phase by constituents originating from the seminal vesicles (Lundquist, 1949; Lindholmer, 1973, Eliasson and Johnsen, 1986). Disruption of this normal ejaculatory sequence can result in poor sperm motility (Mann and Lutwak-Mann, 1981). Human semen coagulates rapidly following ejaculation and then normally liquefies within 5 to 20 minutes (Tauber et al., 1980). The substrates responsible for the coagulation reaction are associated with the secretions of the seminal vesicles whereas the liquefying activity is associated with prostatic secretions (Mann and Lutwak-Mann, 1981). A very exhaustive description of the constituents of seminal plasma, in various species, can be found in the literature (Mann and Lutwak-Mann, 1981).

In the present section, we will focus our attention on compounds that are either very specific to seminal plasma or others that are also be expressed in other tissues or fluids, but are present in semen at unusually high concentrations. These substances are thus more likely relevant to the reproductive process.

1.5.2 Ions and small organic molecules

Sodium and chloride are the main ions in seminal plasma, but their concentrations remain well below that of blood plasma (Mann and Lutwak-Mann, 1981; Kavanagh, 1985). Potassium, on the other hand, is present in a relatively high concentration, and thus with multiple other small organic molecules, it is thought to compensate for the osmolality deficit (Mann and Lutwak-Mann, 1981). Calcium and magnesium ions are present in seminal plasma at concentrations similar to those of blood plasma (Mann and Lutwak-Mann, 1981; Coffey,

1994). An unusual feature of seminal plasma is the abundance of zinc ions which averages 2 mM (Mann and Lutwak-Mann, 1981). This divalent ion originates from the prostate where, of all organs, it is found at the highest concentration (Bertrand and Vladesco, 1921). Its concentration in prostatic secretions can be as high as 7 mM, roughly 20 to 30 times the concentration found in blood plasma (Lynch et al., 1994). Zinc is used as a marker for the secretory function of the prostate gland, and is thought to be mainly secreted as a citrate chelate although this process is not well defined (Kavanagh, 1994). Its function is not well established, but some studies have proposed possible roles. Zinc is known to be important for the maintenance of chromatin stability in the sperm nucleus (Kvist et al., 1990). Following ejaculation, zinc ions bind to protein ligands originating from the seminal vesicles (Arver, 1980; Bjorndal and Kvist, 1990). These zinc ligands may, in this way, affect the zinc complexed to sperm chromatin by competing with cells for seminal zinc and thus influence sperm function (Kjellberg et al., 1992). Fair and Wehner (1976) have also shown that zinc may have an antibacterial effect. Recently, a role for zinc in regulating hamster sperm capacitation has been suggested (Andrews et al., 1994).

Citrate is one of the major anions of seminal plasma and is present at concentrations varying between 13 and 50 mM (Kavanagh, 1994; Lynch et al., 1994). The human prostate is the major source of citrate which is also used as a marker for the gland. Prostatic secretions have been shown to contain up to 180 mM of citrate (Kavanagh, 1994). It is believed to be actively synthesized in the gland rather than simply concentrated from blood citrate (Costello and Franklin, 1991). Citrate is recognized mainly for its important role in maintaining the osmotic equilibrium of the prostate, and it is a powerful zinc chelator in the prostate (Mann and Lutwak-Mann, 1981; Coffey, 1994; Kavanagh, 1994).

Fructose, the essential reducing sugar in seminal plasma, is actively secreted by the seminal vesicles and is used as a marker for the gland's secretory function (Mann and Lutwak-Mann, 1981). Fructose is synthesized from glucose and its concentration ranges between 6 and 60 mM in seminal plasma (Aumüller and Riva, 1992). It represents the main metabolizable substrate in semen (Mann and Lutwak-Mann, 1981). In addition, fructose may be involved in the phenomenon of semen coagulation (Montagnon et al., 1985). Other sugars such as glucose, sorbitol, inositol, ribose and fucose are also present in seminal plasma at low concentrations (Mann and Lutwak-Mann, 1981; Coffey, 1994)

Phosphorylcholine is a highly specific substrate for the enzyme prostatic acid phosphatase present in semen (Seligman et al., 1975). This compound represents the major source of bound choline in seminal plasma, but its exact function in semen remains unknown.

Spermine, a basic aliphatic polyamine, is present at high concentrations in human seminal plasma (1 to 3 mM) and originates from the prostate (Setchell and Brooks, 1994). Interest in spermine and related polyamines originates from studies showing a correlation between changes in polyamine levels and the induction of cell growth (Coffey, 1994). However, in semen, its exact biological function remains speculative, although there may exist a relationship with sperm count and motility (Fair and Parrish, 1981). Free inorganic phosphate released by the seminal acid phosphatase is known to interact with spermine and trigger the formation of spermine phosphate crystals in seminal plasma (Setchell and Brooks, 1994).

Seminal plasma is also the richest source of prostaglandins of all body fluids (Mann and Lutwak-Mann, 1981; Aumüller and Riva, 1992). Multiple members of the prostaglandin family are present in seminal plasma, ranging in concentration between 100 and 300 µg/ml (Coffey, 1994). The name prostaglandin is derived from the initial belief that these

compounds originated from the prostate (von Euler, 1934). However, the seminal vesicles were subsequently found to be the source of the prostaglandins, hence the misnomer (Eliasson, 1959). These molecules are known for their potent pharmacological effects but their role in semen is poorly understood. Although there is controversy concerning the role of prostaglandins in stimulating sperm motility, it is currently believed that they may have effects on cervical mucus and vaginal secretions, thus promoting sperm transport in the female reproductive tract (Clavert et al., 1985; Aümuller and Riva, 1992; Coffey, 1994). The prostaglandins are also believed to have immunosuppressive activity (Kelly, 1991).

Seminal plasma also contains a broad range of free amino acids, and their concentration is known to increase during semen liquefaction due to extensive proteolysis (Aümuller and Riva, 1992; Setchell and Brooks, 1994). Numerous lipids are also present in seminal plasma, the most abundant ones being cholesterol and phospholipids (Scott, 1945; White et al., 1976). The predominant phospholipids are phosphatidylserine and sphingomyelin (Nissen and Kreysel, 1988). Lipids in seminal plasma may interact with the sperm plasma membrane (Eddy and O'Brien, 1994). Finally, many reducing substances such as ascorbic acid, ergothioneine, hypotaurine and uric acid, which originate from different glands depending on the species, are present in seminal plasma (Mann and Lutwak-Mann, 1981; Setchell and Brooks, 1994).

1.5.3 Proteins in seminal plasma

Human seminal plasma contains a wide variety of proteins with a total concentration of 35-55 mg/ml (Lizana and Eneroth, 1983). Only a minor proportion of these proteins are abstracted from serum, and most are actively secreted by the various accessory glands and are specific

for semen. Many proteins have been identified and characterized based on activities or properties reminiscent of known proteins in other tissue and systems. However, other proteins have also been found to be specific to semen and likely play an active role in the reproductive process.

1.5.3.1 Enzymes

Among the proteins identified in seminal plasma, several are enzymes. In human seminal plasma, the presence of a γ-glutamyl transferase (Heite and Wetterauer, 1977), various ribonucleases (Lee et al., 1983) and a sorbitol dehydrogenase (Ibarra et al., 1982) have been reported. In rodents, a transglutaminase catalyzing the covalent cross-linking of seminal vesicle proteins (Williams-Ashman, 1984) and a sulfhydryl oxidase, which catalyzes the oxidation of thiol groups in the presence of oxygen to form disulfides are present (Seitz and Aümuller, 1990). An acid phosphatase that catalyzes the hydrolysis of phosphate ester-containing substrates under acidic conditions was reported in both rat (Paul and Richardson, 1969) and dog prostates (Saini and Van Etten, 1978). In humans, the enzyme prostatic acid phosphatase has a long history of use as a tool in the monitoring of prostatic malignancies (Lilja and Abrahamson, 1988) but its physiological function has not been well established. It has been shown to possess phosphatase activity toward tyrosine-phosphorylated proteins (Lin and Clinton, 1986) and may thus have a role in regulating the phosphorylation state of sperm membrane proteins known to control sperm fertilizing capacity (Naz et al., 1991). In prostate cancer, it might be involved in the progression of tumors or metastasis (Chevalier et al., 1988; Ishibe et al., 1991). Finally, several other enzymes and hydrolases including a lactic dehydrogenase, phospholipases and oxidoreductases are present

in seminal plasma and have been described elsewhere (Aümuller and Seitz, 1990; Coffey, 1994).

1.5.3.2 Proteases

Numerous proteolytic enzymes are present at high concentration in seminal plasma (Mann and Lutwak-Mann, 1981). An arginine esterase of prostatic origin is present in very high concentration in dog seminal plasma (Chapdelaine et al., 1984). Yin et al. (1990) reported the presence of multiple gelatinolytic proteinase activities in human seminal plasma and showed that most of them originated from the prostate. Some of the proteases identified include plasminogen activator (Hisazumi, 1970), a collagenase-like peptidase (Lukac and Koren, 1979), angiotensin-converting enzyme or kininase II (Miska et al., 1988), a zinc-dependent peptidase (Laurell et al, 1982), seminin (Syner et al., 1975; Tauber et al., 1980) and a basic carboxypeptidase (Skidgel et al., 1988).

Prostate-specific antigen (PSA) is another seminal enzyme that has attracted a lot of interest since its introduction in clinical practice as a tumor marker in the monitoring of benign and malignant prostatic growth (Oesterling, 1991). PSA was originally isolated from seminal plasma and identified as a prostate-specific protein (Wang et al., 1979). However, recent evidence using ultrasensitive immunoassays demonstrates low levels of its expression in other tissues as well (Diamandis, 1995). The protein was detected in human breast cancer tissue (Diamandis et al., 1994), in the endometrium (Clements and Mukhtar, 1994), in the milk of lactating women (Yu and Diamandis, 1995) and in various tumors originating from different tissues (Levesque et al., 1995). PSA is secreted in semen, by the epithelial cells of the normal and diseased prostate, at a concentration of 0.2-5 mg/ml (Oesterling, 1991). Its complete

primary structure has been determined from protein chemical analysis and from its cDNA sequence (Watt et al, 1986; Lundwall and Lilja, 1987; Schaller et al., 1987). PSA is a single chain, 33 kDa serine protease with high homology to the kallikrein family of processing enzymes and is one of the most abundant proteins in human prostatic secretions (Watt et al., 1986; Schaller et al., 1987; Lundwall and Lilja, 1987; Lilja and Abrahamson, 1988). Contrarily to other members of the kallikrein family which display trypsin-like activity, the primary structure of PSA allows to predict a chymotrypsin-like activity. This prediction is supported by experimental evidence using synthetic substrates and various protein substrates (Watt et al., 1986; Akiyama et al., 1987; Christensson et al., 1990), and by computer modeling of the three-dimensional structure of the substrate specificity pocket of PSA (Vihinen, 1994). PSA is believed to hydrolyze the main structural protein components of semen coagulum after ejaculation (Lilja, 1985; Lilja et al., 1989; McGee and Herr, 1987; 1988).

The gene and the cDNA coding for another kallikrein protease known as the human glandular kallikrein-1 (hGK-1 or hK2) has been cloned and characterized (Schedlich et al., 1987; Young et al., 1992). The transcript of this gene is expressed at relatively high levels in the prostatic epithelium (Chapdelaine et al., 1988) but there is yet no definite evidence for the secretion of the protein in semen (Paradis et al., 1989). According to its cDNA sequence, the enzyme is highly homologous to PSA but should display trypsin-like substrate specificity. Recently, a novel serine protease, prostasin, has been isolated from seminal plasma and characterized (Yu et al., 1994). It has an apparent molecular weight of 40 kDa, displays trypsin-like activity and is expressed in epithelial and ductal cells of the prostate. It also seems present at lower levels in other tissues and in urine (Yu et al., 1994).

Until now, the majority of seminal proteases characterized have been associated with the extensive protein proteolysis occurring in semen after ejaculation (Mann and Lutwak-Mann, 1981). Besides contributing to the coagulum liquefaction, proteolysis of seminal proteins may also contribute to reduce the antigenic load that semen proteins represent in the vagina (Szcesi and Lilja, 1993). It is also possible that some proteases act on sperm proteins and thus modulate their fertilizing ability or other functional parameters.

1.5.3.3 Protease inhibitors

Since numerous proteases are present at high concentration in seminal plasma, it is natural to suspect that regulatory mechanisms must also exist to control their activity. Unregulated proteolytic activity in semen could result in damage to spermatozoa. It is thus very unlikely that all these proteases are free to act at all time in the reproductive tract or in semen. Accordingly, seminal plasma has been found to be a rich source of various protease inhibitors. The high molecular weight proteinase inhibitors α_1-antitrypsin and α_1-antichymotrypsin are present in seminal plasma (Schill, 1976). The α_1-antichymotrypsin can bind to PSA and regulate its activity (Christensson et al., 1990). In addition, protein C, a member of the serine protease inhibitors (serpins) was detected at high concentration in seminal plasma (Laurell et al., 1992). It appears to originate mainly from the seminal vesicles but also to a lesser extent from the testis, epididymis and prostate. This protease inhibitor might be regulating the activity of PSA, the hGK-1-gene product, and acrosin (Laurell et al., 1992; Christensson and Lilja, 1994). In addition, a family of trypsin inhibitors and a low molecular weight cysteine protease inhibitor have also been described in human semen (Schiessller et al., 1976; Minakata et al., 1986). Beside possible effects on seminal proteases, many of the protease

inhibitors present in seminal plasma are believed to inactivate the sperm acrosomal protease acrosin, released from damaged spermatozoa and to protect spermatozoa, from the various proteases present in the secretions of the female reproductive tract (Aümuller and Seitz, 1990).

1.5.3.4 Immunosuppressants

Proteins with immunosuppressive properties have been reported in seminal plasma (Marcus et al., 1987). Their presence probably reflects the need to avoid sensitization of the female to sperm antigens following intercourse and to protect spermatozoa from potential damage caused by cells of the immune system (James and Hargreave, 1984). Lactoferrin is suspected to provide immunotolerance of the endometrium against spermatozoa (Wichmann et al., 1989). The potential mechanisms for immune response modulation have been described by James and Hargreave (1984). One possibility is the protection of spermatozoa against antibody-mediated destruction provided by proteins of seminal plasma which can interact with the Fc portion of immunoglobulins (Witkin et al., 1983; Thaler et al., 1989; Liang et al., 1991). However, the other side of the coin suggests that the same factors may also have detrimental effects, by decreasing defense mechanisms against the appearance of genital tract tumors, and facilitating the transmission of infectious agents (Ablin et al.,1980; James and Hargreave, 1984; Marcus et al., 1987; Kelly, 1991). In spite of the presence of immunosuppressants, various immunoglobulins are detectable in seminal plasma but their concentrations remain below those found in blood plasma (Coffey, 1994). They are believed to originate from the accessory glands.

1.5.3.5 Structural proteins and semen coagulation-liquefaction

In many mammalian species, semen is known to coagulate into a gelatinous mass after ejaculation (Mann and Lutwak-Mann, 1981). The seminal vesicles are the source of coagulating substances in most species (Mann and Lutwak-Mann, 1981). Interestingly, dogs and cats lack seminal vesicles, and their semen does not coagulate (Tauber and Zaneveld, 1981). The phenomenon of semen coagulation has been extensively characterized in rodents where formation of a solid copulatory plug, which does not readily liquefy, takes place in the vagina (Williams-Ashman, 1984). This plug is believed to prevent the outflow of semen and avoid additional fertilization by non-dominant males (Aumüller and Seitz, 1990). The vaginal plug is formed by extensive covalent bond formation between basic protein substrates originating from the seminal vesicles. This cross-linking of proteins occurs via a reaction between the ϵ- amino group of lysine residues and the γ-glutamyl groups catalyzed by a transglutaminase originating from the coagulating gland (Williams-Ashman et al., 1972; Williams-Ashman, 1984; Fawell and Higgins., 1987). While in the guinea pig a single protein appears to be the precursor of the cross-linked clot (Notides and Williams-Ashman, 1967), several proteins participate in the same phenomenon in rat seminal plasma (Williams-Ashman et al., 1980). The major substrate for the transglutaminase in rat semen appears to be the seminal vesicle secretory protein-II (SVS-II) (Wagner and Kistler, 1987). This protein forms disulfide-linked complexes in the lumen of the seminal vesicle (Wagner and Kistler, 1987) through an oxidation reaction that may be catalyzed by a sulfhydryl oxidase present in the seminal vesicle (Ostrowski and Kistler, 1980). The proteins involved in the plug formation have similar amino acid compositions which are unusually rich in lysine, histidine, serine, and glutamine/glutamic acid (Wagner and Kistler, 1987).

In the boar, a different mechanism involving interactions between sialomucoproteins derived from the Cowper's gland and proteins from the seminal vesicle appears to be responsible for the coagulation of semen (Mann and Lutwak-Mann, 1981).

Following ejaculation, human semen spontaneously coagulates into a semi-solid gelatinous mass which then liquefies within 5 to 20 minutes (Amelar, 1962; Tauber and Zaneveld, 1981). This coagulum appears as a dense network of narrow and long fibers that are approximately 0.15 μm thick (Zaneveld et al., 1974). The major structural components of human semen coagulum have been described as disulfide-linked complexes of a predominant 52 kDa protein, semenogelin, and two less abundant semenogelin-related proteins of 71 and 76 kDa, all originating from the seminal vesicle secretions (Lilja and Laurell, 1984; 1985). Fibronectin, another major secretion of the human seminal vesicles, also appears to be incorporated in the coagulum structure with semenogelin (Lilja et al., 1987). The primary structures of semenogelin and semenogelin-related proteins have been elucidated (Lilja et al., 1989; Lilja and Lundwall, 1992). The 71 kDa semenogelin-related protein (or semenogelin II) is highly homologous (80%) to the 52 kDa semenogelin (or semenogelin I), the main difference being the presence of an extended C-terminal, while the 76 kDa protein appears to be a glycosylated form of the 71 kDa polypeptide (Lilja and Lundwall, 1992). Whereas semenogelin is expressed exclusively in the seminal vesicles, transcripts related to the 71-76 kDa semenogelin-related proteins have been detected in the epididymis (Lilja et al., 1989). These proteins contain various internally repeated units but do not possess significant homology to other known proteins. However, significant sequence similarity exists in the signal peptide and the 3'-end untranslated nucleotides of the cDNA of semenogelin and semenogelin-related proteins and that of both rat seminal vesicle secretory protein II and

guinea pig seminal vesicles protein GP1, the major seminal clotting protein in these species (Lilja and Lundwall, 1992; Lundwall and Lazure, 1995).

The exact molecular mechanism responsible for the gelation of these human seminal proteins remains unclear but some hypotheses have been proposed. Chaistitnavitch and Boonsaeng (1983) observed acceleration of semen liquefaction in the presence of the reducing agent 2-mercaptoethanol. However, the interactions between proteins that are responsible for coagulation do not appear to be mediated by disulfides or other covalent bonds since the coagulum structure is unaffected by reducing agents but the coagulum can be readily solubilized in 2-3 M guanidine-HCl (Lilja and Laurell, 1985). Moreover, in contrast to rodent species, no transglutaminase activity can be detected in human semen (Lilja and Laurell, 1985) although a novel transglutaminase cDNA was recently cloned from a human prostate cDNA (Grant et al., 1994). The mechanism responsible for semen coagulation is different from the one involved in blood coagulation since factor XII, fibrinogen, and prothrombin are all absent from seminal plasma (Mann and Lutwak-Mann, 1981). Lilja and coworkers (1987) have proposed that prostatic components may induce a polymerization reaction between the semenogelins and fibronectin. Alternately, based on observations that heavy metals can prevent semen liquefaction while EDTA enhances it (Lukac and Koren, 1979), Polak and Daunter (1989) suggested that interactions between glycoproteins and metal ions are responsible for coagulum formation. Another model proposed a reticulation of proteins by formation of stable links between fructose and monomer vesicular proteins in the formation of semen coagulum (Montagnon et al., 1985; 1990).

Semen liquefaction, on the other hand, is thought to be induced by proteolytic degradation of the structural protein components, since the disappearance of the gel structure parallels

fragmentation of semenogelin and semenogelin-related proteins (Lilja, 1985; Lilja et al., 1987; McGee and Herr, 1988). A collagenase-like activity (Lukac and Koren, 1979), and the enzymes seminin (Tauber et al., 1980) and plasminogen activator (Hisazumi, 1970) were originally associated with this activity. However, recent evidence suggests that PSA plays the major role in degrading the structural components of the coagulum (Lilja, 1985; Lilja et al., 1987; McGee and Herr, 1988; Lee et al., 1989). PSA was shown to degrade the predominant coagulum proteins in a manner identical to their degradation in liquefying semen. Fragmentation of these proteins parallels semen liquefaction and the progressive release of spermatozoa (Lilja and Laurell, 1984; 1985; McGee and Herr, 1987). Various polypeptides isolated from seminal plasma appear to be derived from the proteolytic degradation of semenogelin (Li et al., 1985; Schneider et al., 1989; Lilja et al., 1989).

1.5.3.6 Proteins that modulate sperm function

The presence in seminal plasma of a variety of proteins that are specific to that fluid suggests that some of these are likely to interact with spermatozoa after ejaculation and modulate their function. A decapacitating action (Bedford, 1983), a role in sperm binding to the zona pellucida (Topfer-Peterson, 1994), and in the inhibition of the acrosome reaction (Reddy et al., 1982; Han et al., 1990), have been associated with components of seminal plasma. In addition, various protease inhibitors present in seminal plasma are believed to bind to spermatozoa and prevent premature activation of the sperm acrosin (Aumüller and Seitz, 1990). However, only a few of the factors that may affect sperm function have been characterized.

An antifertility protein has been isolated from seminal plasma (Audhya et al., 1987). This 200

kDa protein can inhibit, in a reversible manner, the penetration of capacitated mouse spermatozoa through the zona pellucida without influencing sperm motility or acrosome reaction. Caltrin is a low molecular weight calcium transport inhibitor protein that was purified from bovine seminal plasma (Rufo et al., 1982). Seminalplasmin, a protein that antagonizes the function of calmodulin (Gietzen and Galla, 1985) was originally isolated from bull seminal plasma and subsequently found to be identical to caltrin (Lewis et al., 1985). Caltrin binds specifically to the acrosome and the principal part of the tail of bovine spermatozoa (San Agustin et al., 1987). Caltrin proteins have also been identified in the seminal fluid of the guinea pig, rat and mouse (Coronel et al., 1992). Since calcium uptake by spermatozoa is important in modulating hyperactivation and the acrosome reaction, caltrin is believed to prevent premature activation of these processes in the female reproductive tract (Lardy et al., 1988). Once spermatozoa get in the vicinity of the fertilization site, factors present in the oviduct may modify caltrin and transform it into an enhancer of calcium uptake (Coronel et al., 1992).

Bovine seminal fluid contains a family of predominant and closely related proteins that are secretory products of the seminal vesicles. These proteins were originally purified from bovine seminal plasma and named BSP-A1, BSP-A2, BSP-A3 and BSP-30 kDa (Manjunath et al., 1987; Manjunath and Sairam, 1987). BSP-A1/-A2/-A3 have molecular masses between 15-and 16.5 kDa, while BSP-30 kDa has a mass of 28-30 kDa. One particularity of these proteins is that BSP-A1/-A2/-A3 all contain duplicate homologous domains that are similar to type-II structures found in the gelatin binding domain of fibronectin (Seidah et al., 1987), and other proteins (Therien et al., 1995). This specific domain can interact with collagen (Manjunath et al., 1988), apolipoprotein A1 (Manjunath et al., 1989), heparin (Chandonnet

et al., 1990) and calmodulin (Manjunath et al., 1993). BSP proteins have also been shown to bind to the plasma membrane of bull spermatozoa (Manjunath et al., 1988; 1994). The nature of this interaction has been clarified by studies demonstrating specific binding of BSP-A1/-A2/-A3 to phospholipids containing the phosphorylcholine moiety (Desnoyers and Manjunath, 1992, 1993). BSP proteins localize over most of the surface of ejaculated spermatozoa and they can be specifically displaced by phosphorylcholine (Manjunath et al., 1993). Overall, these results suggest that BSP proteins are involved in lipid transport and plasma membrane modifications known to occur during sperm capacitation and acrosome reaction (Chandonnet et al., 1990).

Upon ejaculation, BSP proteins are believed to bind to phospholipids on sperm surface and act as decapacitant factors. During transit through the female reproductive tract, these proteins may be removed from the sperm surface along with certain phospholipids. This would result in destabilization of the plasma membrane, activation of phospholipase A2 and induction of the membrane fusion occurring during the acrosome reaction. This hypothesis is reinforced by recent findings showing that BSP proteins inhibit phospholipase A2 (Manjunath et al., 1994) and modulate the heparin-induced capacitation of bovine spermatozoa (Therien et al., 1995).

Finally, BSP proteins have also recently been shown to bind to insulin-like growth factor-II (IGF-II) (Desnoyers and Manjunath, 1994). However, there is no sequence homology between BSP proteins and any of the known IGF-binding proteins. The IGF-II binding domain of BSP proteins appears different from the phosphorylcholine binding domain, suggesting that BSP proteins could serve as IGF-II binding site on the sperm membrane and modulate the activity of this growth factor.

The boar spermadhesins are another family of proteins that were originally isolated from boar spermatozoa as sperm proteins that display zona pellucida binding activity. Several low molecular weight proteins (12-15 kDa) were purified from acid extracts of ejaculated boar spermatozoa (Jonakova et al., 1991; Sanz et al., 1992a). However, these polypeptides were later found to originate from the seminal vesicles and to coat spermatozoa following ejaculation (Jonakova et al., 1991; Sanz et al., 1992a). The N-terminal amino acid sequence of these proteins showed the common presence of the amino acids alanine, glutamine (or tryptophan) and asparagine and were thus coined AQN-1, AQN-2, AQN-3, AWN-1, AWN-2. In subsequent studies, their complete primary structure was reported (Sanz et al., 1991; 1992b). All these proteins show high homology to each other (40-60%), but no significant homology to other proteins in the databases. The original preparation of AQN-2 was found to be composed of two different polypeptides: a glycosylated form of AQN-3 (Calvete et al., 1993) and PSP-I, a porcine seminal plasma protein whose function is unknown (Rutherford et al., 1992; Kwok et al., 1993).

The spermadhesins display zona pellucida binding activity *in vitro* through a carbohydrate-mediated mechanism (Calvete et al., 1992). These proteins can also inhibit acrosin activity and bind to heparin and serine protease inhibitors (Sanz et al., 1992c; 1993). Modifications to the glycosylation state of spermadhesins may provide a way to regulate their binding properties. Overall, these proteins are believed to act at two different levels of sperm function. They may modulate sperm capacitation through their glycosaminoglycan binding property and may also be involved in sperm-egg recognition and binding through their affinity for oligosaccharide found on zona pellucida proteins (Calvete et al., 1993). All spermadhesin members become coated on spermatozoa following ejaculation and localize over the anterior

part of the sperm head (Calvete et al., 1992). A recent study showed that most of these bound proteins are released upon incubating spermatozoa under capacitating conditions *in vitro,* suggesting a potential role as decapacitating factors (Dostalova et al., 1994). It is suspected that the remaining tightly bound proteins are involved in zona binding.

The BSP and spermadhesin proteins are multimember families of closely related proteins that appear to have multiple activities. Whether all of the proposed activities are relevant physiologically and can affect sperm function remains to be established. However, it is tempting to speculate that their mere abundance in semen and the level of redundancy in each family may reflect the importance of these factors in the reproductive process.

1.5.4 Factors that modulate sperm motility

Although spermatozoa acquire the potential for motility during epididymal transit, they are maintained in an immotile state in the epididymis (Eddy and O'Brien, 1994). Different factors are believed to be responsible for this phenomenon. Immobilin, a protein found in epididymal fluid of the rat, keeps spermatozoa immotile through a viscoelastic mechanism (Usselman and Cone, 1983). In the bull, the low extracellular pH in epididymal fluid maintains spermatozoa immotile (Acott and Carr, 1984; Carr et al., 1985). Progressive sperm motility is initiated at the time of ejaculation, when epididymal spermatozoa come into contact with seminal plasma components (Eliasson and Johnsen, 1986). While addition of human seminal plasma at a final concentration of 8 % was shown to initiate the progressive motility of epididymal spermatozoa (Lindholmer, 1974), prostatic and seminal vesicle secretions have divergent effects. The fluid obtained from the initial fraction of the ejaculate, which is enriched in prostatic components, has very beneficial effects for the induction of motility at a

concentration of 6-8% (Lindholmer, 1974). Moreover, prostatic secretions at a concentration of 1 to 5% induce motility of epididymal spermatozoa (Eliasson and Jonhsen, 1986). On the other hand, seminal vesicle secretions seem to have a sperm motility inhibiting effect. Sperm motility was found to be significantly lower in the last fractions of the ejaculate, which are enriched in seminal vesicle components, than in the first fractions and continued to decrease in a time-dependent manner (Lindholmer, 1973; Lindholmer, 1974).

Different factors are thought to be involved in the activation of sperm motility at ejaculation. An increase in extracellular and intracellular pH appears to activate sperm motility (Acott and Carr, 1984; Carr et al., 1985). The intracellular second messengers cAMP and calcium are important factors in the initiation and maintenance of sperm motility and seminal plasma components that affect the levels of these messengers will thus influence sperm motility (Tash, 1990). The presence of bicarbonate in seminal plasma activates sperm adenylyl cyclase, resulting in an increase in intracellular cAMP concentrations (Okamura et al., 1985). In turn, this nucleotide can activate cAMP-dependent protein kinase, which may phosphorylate proteins that affect the initiation and maintenance of motility (Tash and Means, 1983; Tash, 1990). Support for this model comes from the finding that bicarbonate concentration is correlated with sperm motility (Okamura et al., 1986). In addition, prostaglandins and steroids, which are present in seminal plasma, are known to control cAMP levels in other tissues, and could also affect sperm motility through a similar effect (Garbers and Kopf, 1980). Calcium on the other hand, may affect sperm motility through pathways involving calmodulin (Tash, 1990). Thus, the low molecular weight components in seminal plasma (seminalplasmin/caltrin) that inhibit calcium uptake by spermatozoa are believed to affect sperm motility (Rufo et al., 1982; Shivaji, 1988). In addition, relaxin, and a major

protein in bovine seminal plasma are believed to be involved in the initiation of sperm motility (Lessing et al., 1985; Aumüller et al., 1988)

Paradoxically, different factors identified in seminal plasma were found to have inhibitory effects on sperm motility (Baas et al., 1983; Kononov et al., 1991). Mouse seminal vesicle components of 12-24 kDa can also inhibit sperm motility (Peitz, 1988). In boar seminal plasma, a 5700 Da polypeptide was found to inhibit sperm motility (Strzezek et al., 1992). Jeng and coworkers (1993) have isolated two similar proteins from porcine seminal plasma that display dose-dependent sperm motility inhibiting properties that can be reversed in the presence of porcine follicular fluid. These same proteins were also found to inhibit the proteolytic activity of chymotrypsin (Jeng et al., 1993). More recently, low molecular weight components in bovine seminal plasma showed a dose-dependent inhibition of sperm motility (Al-Somai et al., 1994a). These components appear to be derived from high molecular weight aggregates present in seminal plasma that can be dissociated in citrate or acidic solution. The dissociation process is reversible and aggregates will reform under neutral conditions (Al-Somai et al., 1994a). The aggregation process appears to be mediated through ionic and hydrophobic interactions (Hameed et al., 1991) and sperm motility appears to be less affected when the components are found in high molecular weight aggregates. The low molecular weight factors may be related to the dialyzable factors previously reported in bovine semen to reduce sperm motility recovery after thawing frozen sperm (Garcia and Graham, 1987). Partial ion-exchange separation of the components of these aggregates showed that cationic components were especially detrimental to sperm motility under the disaggregated form, whereas anionic components were inhibitory only in the aggregated state (Al-Somai et al., 1994b). However, the mode of action of these substances on spermatozoa remains to be

investigated.

Finally, the seminal plasma of various species investigated were found to contain factors that can inhibit the motility of demembranated spermatozoa (de Lamirande et al., 1983). Since the present study focuses on the isolation, characterization, and molecular processing of one such motility inhibitor, a more detailed description of the current information available on these factors follows.

1.6 Seminal plasma sperm motility inhibitor (SPMI)

Hoffman-Berling (1955) was first to demonstrate that treatment of grasshopper spermatozoa with 50% glycerol results in the loss of their membrane selective permeability and in the arrest of flagellar movement. However, motility was reinitiated by the addition of ATP as long as magnesium was also present in the medium. Gibbons and Gibbons (1972) later showed that excellent motility reactivation was achieved using sea urchin spermatozoa if the non-ionic detergent Triton X-100 was used instead of glycerol to demembranate the cells. This was an important achievement that provided a new, useful, and efficient system to study intracellular, and exogenous, factors that control or affect sperm motility. The main advantage of this model was that it allowed direct access to the axonemal machinery without the interference of the plasma membrane. Later on, this marine sperm model was adapted to permit the demembranation and reactivation of mammalian spermatozoa (Lindemann and Gibbons, 1975). Using this model, Mohri and Yanagimachi (1980) showed that epididymal spermatozoa collected in physiological solutions were readily reactivated following demembranation. On the other hand, the reinitiation of motility of ejaculated and then

demembranated rabbit spermatozoa was not possible (Gagnon et al., 1981). Interestingly, spermatozoa washed on a Ficoll gradient before demembranation, to remove the seminal plasma, reinitiated motility following addition of Mg.ATP (de Lamirande et al., 1983). Moreover, it was also observed that the addition of seminal plasma to demembranated-reactivated epididymal spermatozoa caused an immediate arrest of motility. These results suggested that some component(s) present in seminal plasma interfered with the reinitiation of motility. The seminal plasma of other mammalian species was also found to contain a similar inhibitory activity (de Lamirande et al., 1983). The motility inhibitor does not seem species specific since the seminal plasma from one species can inhibit the motility of demembranated-reactivated spermatozoa from different species. However, it appears to be specific to seminal plasma, since blood plasma and other tissues tested were devoid of inhibitory activity (de Lamirande et al., 1983). The inhibitory factor originates from both the prostate and the seminal vesicles in bulls, rats and rabbits but the highest levels were found in the latter tissue (de Lamirande and Gagnon, 1984). Surprisingly, in bulls the levels of inhibitory activity were higher in seminal plasma than in seminal vesicles. This contrasts with a decrease of activity in seminal plasma that would normally be expected due to dilution of seminal vesicle fluid with other fluids at ejaculation. Moreover, dialysis of bull seminal plasma resulted in the loss of 70% of its inhibitory potency while the dialysate did not contain inhibitory activity by itself (de Lamirande and Gagnon, 1984). On the other hand, when the dialyzed seminal plasma was recombined with the dialysate in the proper proportion, the original activity level was restored. These results suggest that there is a small dialyzable molecule, of yet unknown origin, present in bovine seminal plasma that potentiates the activity of seminal vesicle fluid or of dialyzed seminal plasma.

The inhibition of movement of demembranated-reactivated spermatozoa caused by seminal plasma is reversed by addition of Mg.ATP (de Lamirande et al., 1984). Moreover, as the initial concentration of Mg.ATP in the reactivation medium is increased, before addition of seminal plasma, a larger quantity of seminal plasma is required to inhibit sperm motility (de Lamirande et al., 1984).

The sperm motility inhibitor has been isolated from both boar and human seminal plasma and named seminal plasma motility inhibitor (SPMI) (Iwamoto and Gagnon, 1988a; Iwamoto et al, 1992). In the boar, it appears to be a major protein of seminal plasma. Its molecular mass has been estimated to be 50 kDa by gel filtration under nondenaturing conditions (Iwamoto et al, 1992). However, three different polypeptides of 14, 16 and 18 kDa were observed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). It is a thermolabile protein that is stable between pH 6 and 11, and a potent and specific dynein ATPase inhibitor (Iwamoto et al, 1992). Boar SPMI originates exclusively from the epithelial cells of seminal vesicles (Iwamoto et al., 1993). The cDNA encoding the 14 kDa subunit of boar SPMI has recently been characterized (Iwamoto et al., 1995). Interestingly, its deduced amino acid sequence shows 97% homology to AQN-3, a member of the boar spermadhesin protein family (Sanz et al., 1991). This spermadhesin protein had previously been characterized for its carbohydrate-binding properties (see section 1.5.3.6).

In humans, SPMI was purified 280-fold from seminal plasma, suggesting that it is a minor component of that fluid (Iwamoto and Gagnon, 1988a). It is a very basic protein (pI of 9.1) with a molecular mass estimated at 13 to 15 kDa by nondenaturing gel filtration or 18 to 22 kDa by SDS-PAGE. It is stable over a wide pH range (5 to 10), up to 60°C, and is also a potent dynein-ATPase inhibitor (Iwamoto and Gagnon, 1988a). Beside its effect on

demembranated-reactivated spermatozoa, SPMI can also interfere with the motility of intact spermatozoa (Iwamoto and Gagnon, 1988b). The effect is dose-dependent and causes a progressive decrease in the percentage of motile spermatozoa, the curvilinear velocity and the beat-cross frequency, without affecting the linearity. The effect on the percentage of motility increases with the time of contact between the inhibitor and spermatozoa. SPMI at a concentration of 1800 U/ml can completely inhibit the movement of intact spermatozoa within 5 minutes (Iwamoto and Gagnon, 1988b). Moreover, the presence of seminal plasma during incubation of the purified factor with intact spermatozoa does not compromise the inhibitory effects but rather potentiates it. Human SPMI originates exclusively from the seminal vesicles (Luterman et al., 1991).

While the inhibitory potency of seminal plasma does not correlate with the percentage of motile spermatozoa in that fluid, SPMI may be a contributing factor in cases of infertility caused by poor sperm motility. Experimental support in that favor came from the demonstration that some extracts of spermatozoa obtained from men with poor sperm motility contained factors that prevented the reactivation of motility of highly motile control spermatozoa (de Lamirande et al., 1986). In contrast, none of the control samples extracts demonstrated inhibitory activity. Whether these factors are similar to SPMI remains to be established.

1.7 Rationale and outline of the present study.

Since the secretions of the seminal vesicles account for approximately 60 to 70% of the total volume of the ejaculate (Coffey, 1994), the concentration of SPMI in the seminal vesicle fluid can be expected to be approximately 1.6-fold higher than that of seminal plasma, by a simple concentration effect. The concentration of SPMI antigen as measured by an enzyme-linked immunosorbent assay (ELISA), using an anti-SPMI antiserum, corresponds to this expected value, being 1.54-fold higher in seminal vesicles secretions than in seminal plasma (Luterman et al., 1991). However, when levels of SPMI inhibitory activity in seminal vesicle fluid were quantified, a value 13.6-fold higher than that found in seminal plasma was obtained. This puzzling and unexpected finding of a 9-fold difference in SPMI specific activity between seminal vesicle fluid and seminal plasma thus suggests that SPMI originating from the seminal vesicles is transformed into a less active form as it gets mixed with fluids from the various accessory glands at ejaculation. In the beginning of the present study, no experimental evidence to explain this difference in SPMI activity between seminal vesicle fluid and seminal plasma was available. The rationale underlying the present study was to attempt to elucidate the cause and investigate the mechanism responsible for the difference in SPMI specific activity between seminal vesicle fluid and seminal plasma. While the levels of SPMI activity present in liquefied seminal plasma do not appear to inhibit the motility of intact spermatozoa, the high levels found in seminal vesicle fluid are in the range of SPMI concentrations shown to inhibit intact sperm motility (Iwamoto and Gagnon, 1988b). The change in SPMI activity after ejaculation thus appears essential for spermatozoa to acquire normal motility and allow them to undertake their long journey up the female reproductive tract. Anomalies in the process leading to SPMI inactivation after ejaculation would likely result in very low sperm

motility and associated fertility problems. For this reason, investigating the mechanism underlying this change in SPMI activity after ejaculation appeared of prime importance.

A series of experiments that allowed to elucidate the mechanism explaining this phenomenon were elaborated. In the first part of the present study we provide evidence that SPMI exists in the form of a precursor molecule of higher molecular mass in the seminal vesicle fluid, and that it is rapidly processed after ejaculation into less active polypeptides of lower molecular masses by proteases originating from the prostate. SPMI processing paralleled the phenomenon of semen liquefaction and these original results demonstrated close similarities between SPMI and semenogelin, the predominant structural protein of seminal coagulum. This paved the way for further investigation of possible association of SPMI with semen coagulum components. We could demonstrate in the second part of the study that SPMI is in fact associated with the constituents of the semen coagulum and that semen that fails to liquefy spontaneously contains high levels of SPMI activity that may significantly affect sperm motility. These findings lead to the third part of this study where a novel method to purify the SPMI precursor from seminal vesicle fluid as well as seminal coagulum was developed and its properties were characterized. Finally, the processing of purified SPMI precursor by the serine protease PSA was studied. The results demonstrate that PSA is the main enzyme responsible for SPMI processing. In addition, analysis of the proteolytic fragments allowed mapping of the precursor molecule and characterization of PSA activity toward its major physiological substrate.

Chapter II

Sperm motility inhibitor from human seminal plasma: presence of a precursor molecule in seminal vesicles fluid and its molecular processing after ejaculation

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Preface

The following chapter presents the original experiments developed to investigate the mechanism responsible for the large difference in SPMI activity levels between seminal vesicle fluid and seminal plasma.

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Sperm motility inhibitor from human seminal plasma: presence of a precursor molecule in seminal vesicle fluid and its molecular processing after ejaculation

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Summary

Human seminal plasma contains a protein factor that has the capacity to inhibit the movement of demembranated and intact spermatozoa. This factor 'seminal plasma motility inhibitor' (SPMI) has been shown to originate exclusively from the seminal vesicles. The present results demonstrate that the biological activity of SPMI in semen decreases rapidly from 1000 U/ml, immediately after ejaculation, to 220 U/ml 2 h later. Immunoblots of seminal plasma proteins probed with an antibody against human SPMI, revealed the rapid processing of a predominant 52 kDa SPMI antigen, present in the seminal vesicle secretions. This precursor was degraded initially into intermediate molecular mass fragments of 25–40 kDa, and subsequently into smaller fragments of 17–21 kDa. When seminal vesicle fluid was mixed with prostatic secretions (3 : 1 v/v), proteases present in prostatic secretions were shown to be responsible for processing of the SPMI precursor. Addition of protease inhibitors such as phenylmethylsulphonyl fluoride (PMSF, 5 mM), benzamidine (100 mM) or ethylenediaminetetraacetic acid (EDTA, 5 mM) to the mixture of seminal vesicle and prostate secretions partially prevented the loss of activity of SPMI by 54%, 27% and 9%, respectively. However, the simultaneous addition of PMSF and benzamidine conferred almost total stability to the SPMI precursor activity. These results demonstrate that SPMI exists as a predominant 52 kDa precursor form in the seminal vesicles and is processed rapidly after ejaculation into less active, lower molecular mass forms by one or more serine proteases and/or metalloproteases of prostatic origin which are present in liquefied semen.

Keywords: Protease inhibitors, protein precursor, proteolytic processing, semen, serine proteases, spermatozoa

Introduction

Human seminal plasma is composed of secretions from multiple glands of the reproductive tract, among which the main contributors in terms of volume are the seminal vesicles (60–70%) and the prostate (15–25%) (Eliasson & Johnsen, 1986; Coffey, 1988). Contributions from the testis, the sperm-rich epididymal fluid and from Cowper's and Littre's glands accounts for only 10–15% of the total semen volume (Lundquist, 1949). Human seminal plasma contains factors which are either beneficial or detrimental to sperm motility and which have been associated with prostatic and vesicular secretions, respectively (Lindholmer, 1973, 1974). Upon ejaculation, the secretions from the different accessory glands mix and a coagulum forms spontaneously. The coagulum then liquefies within 5–20 min, through proteolytic degradation of its structural components (Lilja & Laurell, 1985; Lilja *et al.*, 1987).

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The motility of mammalian spermatozoa can be arrested by nonionic detergents and reinitiated subsequently upon addition of Mg.ATP (Lindemann & Gibbons, 1975; Mohri & Yanagimachi, 1980; de Lamirande *et al.*, 1983). Using this model, epididymal spermatozoa collected in physiological solutions are readily reactivated following demembranation (Mohri & Yanagimachi, 1980). de Lamirande *et al.* (1984) observed that the reinitiation of motility is prevented when ejaculated spermatozoa are used. However, once spermatozoa from semen are washed free of seminal plasma, they reactivate normally after demembranation. It was thus suggested that some components of seminal plasma interfered with the reinitiation of motility. The seminal plasma of most mammalian species investigated so far have been shown to contain a sperm motility inhibitor (de Lamirande *et al.* 1983, 1984). The inhibitor is not species-specific since the seminal plasma from one species can inhibit the motility of demembranated–reactivated spermatozoa from a different species (de Lamirande *et al.*, 1983, 1984). However, it is specific to the reproductive system, being absent from all other tissues tested (de Lamirande *et al.*, 1983).

The human sperm motility inhibitor has been purified from seminal plasma and named 'seminal plasma motility inhibitor' (SPMI, Iwamoto & Gagnon, 1988a). It is a very basic protein (pI 9.1) of 18–22 kDa that is stable over a wide range of pH (6–10) and to temperatures of up to 60°C. Besides its effect on demembranated–reactivated spermatozoa, SPMI can also inhibit the motility of intact ejaculated spermatozoa by decreasing the percentage of motile cells, their curvilinear velocity as well as their beat/cross frequency (Iwamoto & Gagnon, 1988b). The observation that the effect of SPMI on demembranated–reactivated spermatozoa can be reversed by increasing amounts of ATP in the assay medium (de Lamirande *et al.*, 1984) and that SPMI can inhibit purified bull dynein ATPase in a dose-dependent manner (Iwamoto & Gagnon, 1988a; Gagnon *et al.*, 1992; Iwamoto *et al.*, 1992), suggests that it may act on demembranated spermatozoa by interfering with function of the dynein arms.

Luterman *et al.* (1991) have shown that human SPMI originates exclusively from the seminal vesicles. The biological activity of SPMI per unit volume of seminal vesicle fluid is 14-fold higher than that of seminal plasma. However, the concentration of SPMI antigen measured by an enzyme-linked immunosorbent assay (ELISA) using anti-SPMI antibodies is only 1.6-fold higher in seminal vesicle fluid when compared to that of seminal plasma (Luterman *et al.*, 1991). This 1.6-fold higher ratio in antigen concentration is probably explained by the simple dilution of the seminal vesicle secretions by the fluids of the other accessory glands at ejaculation. On the other hand, the 8–9-fold difference in specific activity (units of biological activity/μg SPMI antigen) between these two fluids suggests that the SPMI activity present in seminal vesicle secretions decreases after ejaculation. In the present study we attempted to elucidate the possible mechanism accounting for this difference in specific activity. We report that SPMI is present in seminal vesicle fluid as a predominant precursor form of 52 kDa and that after ejaculation it is processed rapidly into less active, smaller molecular forms by proteases of prostatic origin.

Materials and methods

Reagents

Glycine, *N*-2-hydroxyethyl piperazine *N*-2'-ethanesulphonic acid (HEPES), benzamidine, sodium dodecylsulphate (SDS) and dithiothreitol (DTT) were purchased from ICN Biomedicals (Montreal, Canada); 1,10-phenanthroline, Triton X-100 and soybean trypsin inhibitor from Sigma Chemical Co. (St Louis, MO, U.S.A.); acrylamide, β-mercaptoethanol (β-ME), Coomassie blue, nitroblue tetrazolium chloride (NBT) and bromo-chloro-indolphosphate (BCIP) from Bio-Rad Laboratories (Mississauga, Canada); adenosine triphosphate (ATP), phenylmethylsulphonyl fluoride (PMSF), leupeptin, L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK), α_2-macroglobulin and pepstatin A from Boehringer Mannheim (Laval, Canada); Tween-20 and ethylenediaminetetraacetic acid (EDTA) from Fisher Scientific (Montreal, Canada). Percoll was obtained from Pharmacia (Baie d'Urfé, Canada), porcine serum from Gibco BRL (Burlington, Canada), and the alkaline phosphatase-conjugated goat anti-rabbit IgG from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.). All other chemicals were at least of reagent grade.

Collection of seminal vesicle fluid (SVF) and prostatic fluid (PF)

SVF was obtained during surgical procedures on patients undergoing radical prostatectomy (prostate carcinoma, stage B, mean age: 58 ± 2 years, $n = 2$) or at autopsy (accidental death, mean age: 33 ± 9 years, $n = 10$). SVF was collected directly by needle aspiration from the ducts of the ligated gland. PF was obtained exclusively from autopsy specimens. The prostate was dissected free from surrounding tissues and opened longitudinally through the urethra. PF was collected after thorough washing of the gland with 0.9% NaCl, by firmly squeezing the gland, and collecting fluid at the level of the verumontanum. These fluids were diluted in an equal volume of Hepes saline solution (HSS, 25 mM Hepes, 100 mM NaCl) at pH 8.0, to facilitate handling and recovery of proteins, mixed and then centrifuged for 20 min at 10000 *g*. The supernatants were collected and frozen at −70°C until used.

Semen collection and sperm washing

Semen was provided by healthy male volunteers by masturbation into sterile containers after 3 days of sexual abstinence. Two different semen samples were used in each experiment. To measure the early changes in SPMI activity

and molecular forms, one sample was obtained 1–2 min after ejaculation, centrifuged briefly (30 sec at 10 000 *g*) and assayed for biological activity and immunoblotting. The other sample was allowed to liquefy normally at room temperature. Following liquefaction the semen was layered on a discontinuous Percoll density gradient made up of 1.5 ml each of 20%, 40%, and 65% Percoll and 0.2 ml of 95% Percoll buffered in HBS (25 mM Hepes, 130 mM NaCl, 0.5 mM $MgCl_2$ and 14 mM fructose) at pH 8.0. Following centrifugation at 1300 *g* for 30 min, the spermatozoa from the 65–95% Percoll interface and the 95% Percoll layer were recovered and used in the SPMI activity assay.

SPMI activity assay

Percoll-washed human spermatozoa were demembranated in a medium containing 0.1% Triton X-100 , 0.2 M sucrose, 0.025 M potassium glutamate pH 8.0, 0.035 M Tris-HCl pH 8.0 and 1 mM DTT (de Lamirande *et al.*, 1983). After demembranation the motility of spermatozoa was reinitiated by the addition of 0.5 mM Mg.ATP. One unit of SPMI biological activity is defined as the minimal amount of protein that completely inhibits the motility of demembranated–reactivated spermatozoa within 5–10 s in 1 ml of demembranation–reactivation medium.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins from seminal plasma and other fluids were separated by SDS-PAGE according to Laemmli (1970) using the Bio-Rad, Mini Protean II electrophoresis system. The stacking and running gels contained 5% and 15% acrylamide, respectively. Electrophoresis was at a constant current of 20 mA for 1 h. Gels were then stained with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol/10% acetic acid and destained successively in 40% methanol/10% acetic acid and 10% methanol/10% acetic acid.

Immunoblotting and immunodetection

Following electrophoresis, proteins were electrotransferred from slab gels onto Nitroplus nitrocellulose membranes (Micron Separations Inc., Westboro, MA, U.S.A.) for 2 h at 70 V. The transfer buffer was composed of 25 mM glycine, 25 mM ethanolamine, 0.01% SDS and 20% methanol, pH 9.8. After transfer, the membranes were air dried and stored at 4°C until used. Membranes were then first stained in 0.2% Ponceau S in 3% acetic acid to visualize proteins and molecular weight markers. After destaining in 10 mM Tris-HCl, 0.9% NaCl (TBS) pH 7.4, nonspecific sites on the membranes were blocked with 10% porcine serum in TBS containing 0.25% Tween-20 and 0.02% sodium azide (blocking solution) for 1 h at room temperature. The membranes were then incubated for 1 h with the rabbit anti-human SPMI antiserum (Luterman *et al.*, 1991) diluted 1 : 200 in blocking solution. After four successive 10-min washes in TBS containing 0.25% Tween-20, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1 : 2000 in blocking solution. Membranes were then washed four times in TBS containing 0.25% Tween-20. Antigens were visualized by incubation in alkaline phosphatase buffer (100 mM Tris-HCl, 1 mM $MgCl_2$, pH 9.5) containing the substrates NBT and BCIP at 0.1 and 0.05 mg/ml respectively. The reaction was terminated by washing the membrane in distilled water.

Protein determination

The concentration of proteins in the samples was measured with the bicinchoninic acid (Pierce Chemical Co., Rockford, IL, U.S.A.) assay using the procedure described by Smith *et al.* (1985). Bovine serum albumin was used as a standard.

Results

Post-ejaculatory changes in SPMI

SPMI biological activity in semen decreased rapidly with time after ejaculation (Fig. 1). From a level of 1000 ± 5 U/ml within 2.5 min from ejaculation, the activity decreased to 220 ± 20 U/ml after 2 h at room temperature, and it remained stable at this level for up to 3 h. Most of the loss in activity of SPMI occurred within the first 30 min. The electrophoretic pattern of seminal plasma proteins was modified rapidly after ejaculation (Fig. 2a). Immunoblots of the same samples probed with anti-SPMI antibodies revealed the presence of multiple immunoreactive bands of various molecular masses (Fig. 2b). A major immunoreactive protein of 52 kDa corresponding to a predominant

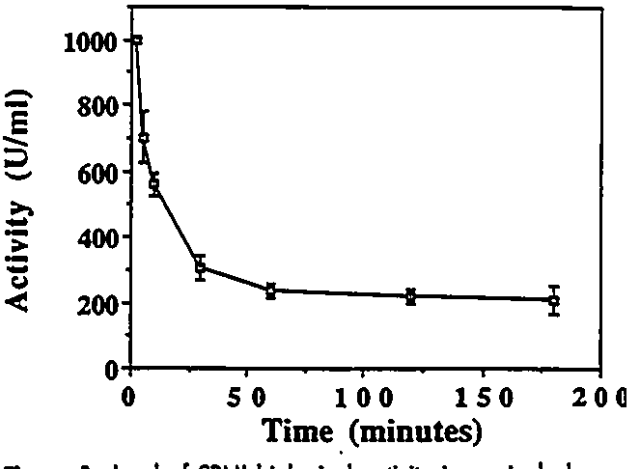

Figure 1. Level of SPMI biological activity in seminal plasma after ejaculation. Semen obtained 1 or 2 min after ejaculation was centrifuged briefly to remove spermatozoa. Seminal plasma was kept at room temperature and SPMI biological activity was measured at several time periods, as described in Materials and methods. The activity is reported as mean ± SEM. (averages of four independent experiments using different ejaculates).

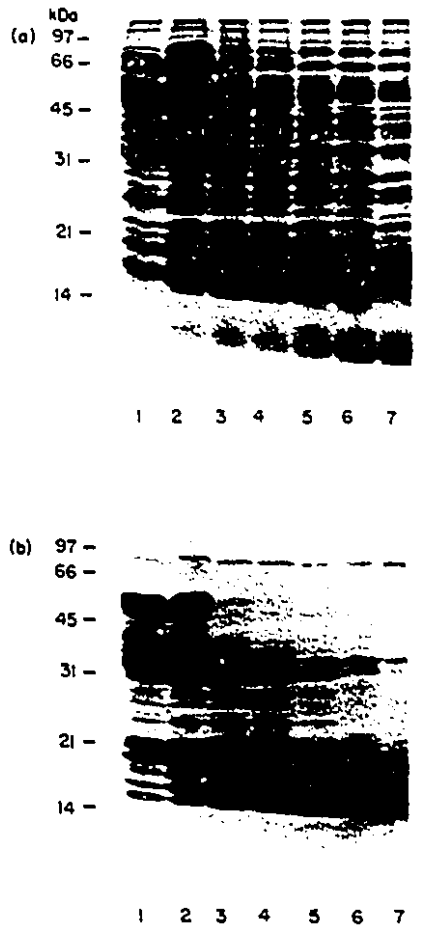

Figure 2. Immunoreactive forms of SPMI in seminal plasma after ejaculation. At each time point of the experiment described in Fig. 1, an aliquot of seminal plasma (30 µg), kept at room temperature, was mixed with SDS sample-buffer, heated to 95°C and analysed by SDS-PAGE (a) and immunoblotting (b). Lanes 1–7 correspond to the time periods in Fig. 1: 2.5 (1), 5 (2), 10 (3), 30 (4), 60 (5), 120 (6) and 180 (7) min after ejaculation.

Coomassie blue-stained polypeptide was observed during the first few minutes after ejaculation. However, its staining intensity decreased rapidly to almost undetectable levels after 10 min, with a concomitant increase of intermediate molecular mass proteins (25–40 kDa) after 5–10 min. These intermediate mass antigens then, in turn, decreased in intensity as they were transformed into still smaller mass peptides of 17–21 kDa found in seminal plasma after 2–3 h. Thus, the decrease in SPMI activity appeared to be associated with the processing of a predominant 52 kDa as well as multiple 25–40 kDa SPMI antigens that are precursors of the lower mass forms present in liquefied seminal plasma.

Effect of prostatic fluid on seminal vesicle SPMI

To determine whether the rapid drop in SPMI biological activity and degradation of the 52 kDa SPMI immunoreactive precursor and associated polypeptides were caused by the action of proteolytic activity present in prostatic secretions, SVF was mixed with prostatic fluid in physiological proportions (3 : 1 v/v). Addition of prostatic fluid to SVF caused a decrease in SPMI activity, from an initial 630 ± 50 U/ml to 240 ± 4 U/ml after incubation for 3 h (Fig. 3). The level of SPMI activity was then maintained at that level for up to 4 h. Most of the drop in SPMI activity occurred within the first 30–50 min. On the other hand, in the absence of prostatic fluid, no change in the level of SPMI activity occurred for up to 4 h. As observed in whole semen, the protein profile of the SVF + prostatic fluid mixture was modified rapidly with increasing time of incubation (Fig. 4a). On immunoblots, SPMI antigens from SVF were characterized, immediately after mixing with prostatic fluid (time 0), by a predominant 52 kDa protein associated with intermediate mass polypeptides present in smaller amounts (Fig. 4b). Only low levels of 17–21 kDa antigens were detected at the initial time. From that point a progressive decrease in the molecular mass of the immunoreactive forms of SPMI paralleled the decrease in biological activity. The major SPMI antigen at 52 kDa was transformed into intermediate mass forms of 25–40 kDa between 2.5 and 10 min. By 15 min the 52 kDa antigen was no longer detected. There was also a progressive increase in the level of 17–21 kDa mass forms between 2.5 and 45 min of incubation. After 4 h the SPMI antigens were almost exclusively within the 17–21 kDa mass range. The whole process occurred in a manner that was very similar to that observed in whole seminal plasma after ejaculation. In the absence of prostatic

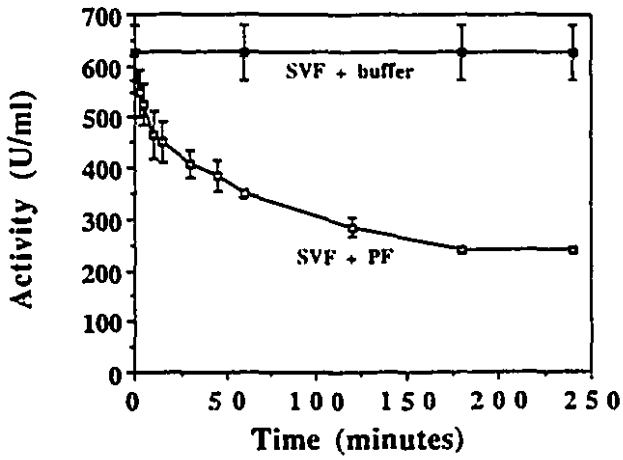

Figure 3. Effect of prostatic fluid on the SPMI activity of seminal vesicle fluid (SVF). The SVF was mixed at room temperature with prostatic fluid (PF, 3 : 1 v/v), or HSS buffer, and SPMI biological activity was measured at different time periods. Prostatic fluid was diluted 2-fold in HSS buffer and had no significant intrinsic SPMI activity (Luterman *et al.*, 1991). The activity is reported as mean ± SEM (averages of three independent experiments using different fluids).

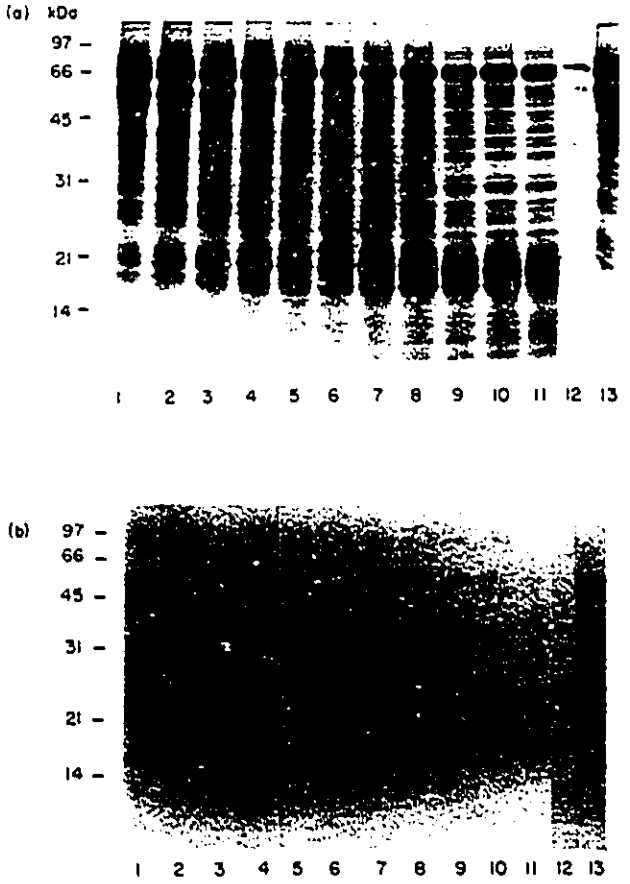

Figure 4. Effect of prostatic fluid on immunoreactive forms of SPMI present in seminal vesicle fluid. At each time point of the experiment described in Fig. 3, an aliquot of the seminal vesicle and prostatic fluid mixture (30 µg), kept at room temperature, was mixed with SDS sample buffer, heated to 95°C and analysed by SDS-PAGE (a) and immunoblotting (b). Lanes 1–11 correspond to the time periods in Fig. 3: 0 (1), 2.5 (2), 5 (3), 10 (4), 15 (5), 30 (6), 45 (7), 60 (8), 120 (9), 180 (10) and 240 min (11) after addition of prostatic fluid to seminal vesicle fluid. Lane 12 = prostatic fluid alone; lane 13 = seminal vesicle fluid in absence of prostatic fluid, after incubation for 4 h.

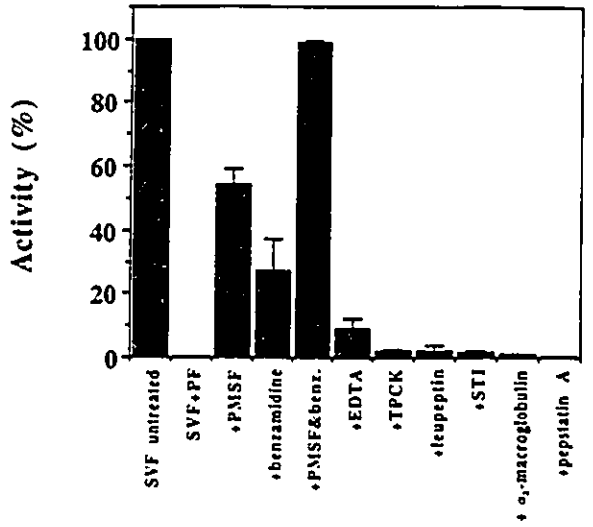

Figure 5. Effect of various protease inhibitors on the degradation of SPMI activity in seminal vesicle fluid (SVF) by prostatic secretions. Prostatic fluid was pre-incubated at room temperature with various protease inhibitors for 60 min. SVF was then added, and the mixture incubated at room temperature. SPMI biological activity was measured 4 h later. The biological activity of untreated SVF is given a relative value of 100%. The SPMI activity remaining after SVF and prostatic fluids were co-incubated for 4 h, in the absence of any protease inhibitor is given a value of 0%. Results are reported as the mean percentage of activity ± SEM relative to these two limits (averages of six independent experiments using different fluids).

fluid, the SPMI immunoreactive forms present in SVF were unaffected for up to 4 h.

Effect of various protease inhibitors on SPMI precursor processing

To investigate further the type of protease involved in processing of the SPMI precursor, various protease inhibitors were incubated with prostatic fluid prior to its addition to SVF. In the absence of any exogenous protease inhibitor, SPMI biological activity decreased rapidly, as described above. On the other hand, the addition of specific protease inhibitors partially prevented the loss of SPMI biological activity caused by prostatic fluid (Fig. 5). PMSF (5 mM), benzamidine (100 mM) and EDTA (5 mM) prevented the loss in biological activity by 54%, 27% and 9%, respectively. When both PMSF and benzamidine were added simultaneously, 100% of the SPMI activity was conserved. Aliquots of prostatic fluid incubated separately with each protease inhibitor were tested in the absence of SVF. All reaction mixtures had no detectable SPMI activity. The effect of these inhibitors on the SPMI molecular mass changes are depicted in Figure 6. In the absence of any protease inhibitor, the predominant 52 kDa SPMI form present in SVF was degraded completely into smaller fragments of 17–21 kDa (Fig. 6b). In the presence of PMSF, the 52 kDa precursor was almost undetectable. However, two predominant intermediate mass proteins of 35 and 33 kDa, as well as three lower mass polypeptides of 21, 20 and 17 kDa, remained after incubation for 4 h. On the other hand, the benzamidine-treated sample showed more extensive degradation of the high molecular mass forms of SPMI and generated only two stable low molecular mass proteins of 20 and 17 kDa; faint reactions occurred between 25 and 35 kDa. The sample treated with both PMSF and benzamidine showed an electrophoretic pattern very similar to that of untreated SVF with the presence of the 52 kDa precursor as well as additional intense reactions at 33–40 kDa. This is

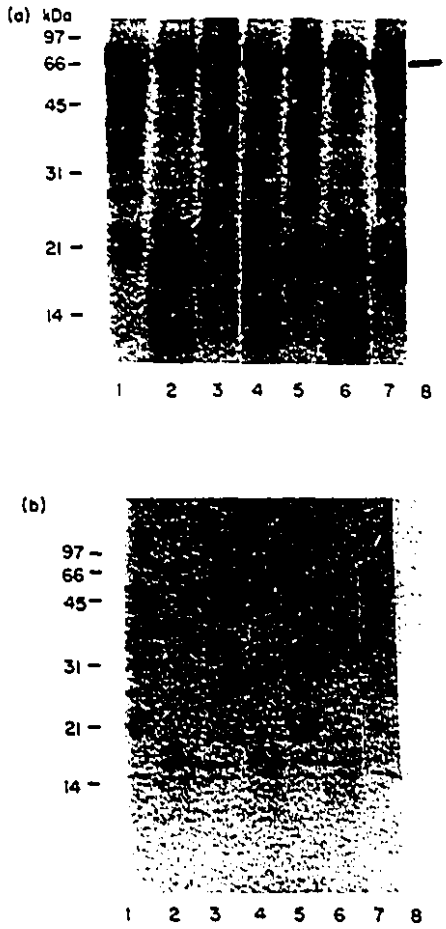

Figure 6. Effect of various protease inhibitors on the degradation of SPMI antigens in seminal vesicle fluid (SVF) by prostatic fluid (PF). The procedure was as described in Fig. 5. After incubation for 4 h, an aliquot (30 μg) was mixed with SDS sample buffer, heated to 95°C and analysed by SDS-PAGE (a) and immunoblotting (b). untreated SVF (1), SVF+PF (2), SVF+PF+PMSF (3), SVF+PF+benzamidine (4), SVF+PF+PMSF+benzamidine (5), SVF+PF+EDTA (6), SVF+PF+1.10-phenanthroline (7), prostatic fluid at concentration used.

consistent with the result of the assay for biological activity which demonstrated total protection against the loss of SPMI activity (Fig. 5).

The zinc chelator 1,10-phenanthroline (50 mM) also provided protection against the processing of the 52 kDa SPMI precursor into smaller molecular mass proteins. The resulting electrophoretic profile was almost identical to that observed using the combination of PMSF and benzamidine (Fig. 5). However, the biological activity of SPMI could not be determined in these samples due to the formation of a coagulated protein complex upon the addition of 1,10-phenanthroline which was solubilized by SDS-PAGE sample buffer but remained insoluble in the medium used for assay of SPMI activity. EDTA was not very effective at protecting SPMI activity and antigens against proteases. The electrophoretic pattern showed extensive protein degradation. Only polypeptides at 20, 17 and 15 kDa, as well as very faint bands around 25–32 kDa, were detected. Samples treated with 1 mg/ml each of TPCK, leupeptin, STI, α_2-macroglobulin or pepstatin A lost biological activity to the same extent as the untreated sample. Extensive degradation into low molecular mass forms (17–21 kDa) was also apparent on polyacrylamide gels and immunoblots. Prostatic fluid alone represented a minor contribution to the total proteins and contained no detectable SPMI antigen.

Discussion

Several mechanisms could account for the difference in the levels of SPMI activity between SVF and seminal plasma; for example, the presence of an activator of SPMI in SVF and its inactivation at ejaculation, when mixing of secretory fluids from the various accessory glands occurs. Alternatively, the presence in seminal plasma of an inhibitor originating from a gland other than the seminal vesicles could explain this difference in activity. However, the results of the present study favour a third mechanism. It is clearly apparent from the immunoblotting results that the SPMI in seminal vesicles is present as a precursor form of higher molecular mass than that found in liquefied seminal plasma (Figs 2b and 4b). The difference in SPMI activity between SVF and seminal plasma is therefore probably explained by the extensive processing after ejaculation of a predominant 52 kDa SPMI precursor molecule, as well as associated 25–40 kDa polypeptides, into the low mass (17–21 kDa) SPMI forms present in liquefied seminal plasma. The results suggest that the 19 kDa SPMI, isolated originally from seminal plasma by Iwamoto & Gagnon (1988a), and to which the SPMI antibody used in the present study was raised, was a degradation product of the 52 kDa SPMI precursor.

The detection of multiple immunoreactive SPMI bands shortly after ejaculation does not appear to be the result of a lack of specificity of the antibody since several bands of proteins clearly remain undetected after reaction with the SPMI antibody. Unrelated protein fluids tested with the antibody did not demonstrate any immunoreactivity (data not shown). A wide mass heterogeneity of SPMI antigens is apparent in SVF (Figs 4b and 6b). Whether this is an artifact resulting from manipulation or collection, or is an intrinsic property of the fluid, remains to be established. However, the detection of multiple antigenically related polypeptides in SVF and seminal plasma has been demonstrated previously by McGee & Herr (1987, 1988). Using the monoclonal antibody MHS-5, generated against the seminal vesicle-specific antigen (SVSA) these authors showed the presence of multiple immunoreactive forms (10–71 kDa) of SVSA in seminal plasma immediately after ejaculation and in native

seminal vesicle secretions. The presence of such heterogeneity (10–15 antigenic polypeptides) in antigen immunodetection in seminal plasma is not so surprising considering the extensive protein processing known to take place after ejaculation (Mann & Lutwak-Mann, 1981). Multiple SPMI and SVSA antigens seem to share several characteristics, including molecular mass of precursors and intermediates, as well as the time-frame of processing. It should be noted that one 15 kDa immunoreactive SPMI polypeptide appears to be present uniformly in all samples, regardless of the time period after ejaculation or incubation of SVF and prostatic fluid. This polypeptide appears to escape the normal proteolytic processing and does not correspond with any major Coomassie blue staining polypeptide. Thus this polypeptide seems to represent a minor SPMI antigen even though it is highly immunoreactive. Another characteristic of the SPMI antibody used was relatively poor staining of the 52 kDa precursor (see in Figs 2 and 4). Since the antibody used was generated from the 19 kDa peptide purified by Iwamoto & Gagnon (1988a), the epitope on the 52 kDa precursor may not be as readily available to the antibody.

The in vitro mixture of SVF and prostatic fluids can closely mimic the events occurring in semen after ejaculation (Figs 1–4). Thus, at ejaculation, when prostate and seminal vesicle fluids mix, the prostatic components induce a rapid degradation of the 52 kDa SPMI precursor and its associated intermediate mass antigens into smaller fragments and therefore reduce its biological activity considerably. The loss of activity and change in molecular mass occurred at a slower pace in experiments in which SVF and prostatic fluids were co-incubated, when compared to that in seminal plasma. This difference results probably from the use of diluted prostatic fluid for the incubation. The degradation of SPMI precursor forms can be prevented by the addition of some protease inhibitors, the concentrations of which were chosen to be in excess of that suggested normally to ensure complete inhibition of their respective proteases. Only PMSF, benzamidine and 1,10-phenanthroline conferred significant protection against the loss of SPMI precursor forms and of biological activity.

Numerous studies have reported proteolytic activities in semen such as a zinc-dependent peptidase (Laurell *et al.*, 1982), neutral protease or seminin (Syner *et al.*, 1975, Tauber *et al.*, 1980), collagenase-like peptidase (Lukac & Koren, 1979), kallikrein-like serine protease (Lilja, 1985) and prostate-specific antigen (PSA; Watt *et al.*, 1986). The observation that two protease inhibitors (PMSF and benzamidine) were necessary to prevent the loss of SPMI biological activity and degradation of the 52 kDa precursor, suggests that at least two proteases are involved in this processing. The first is presumed to be a serine protease which is inhibited by PMSF and the other a protease with properties similar to that of trypsin because of its sensitivity to benzamidine, an analogue of arginine side chain. This hypothesis is reinforced by the observation that the extent of SPMI activity protection was different (54% vs. 27%) for PMSF and benzamidine, and that fragments of different molecular mass were obtained when these two inhibitors were used. It is therefore unlikely that PMSF and benzamidine act on the same protease.

Possible candidates for the proteases involved in SPMI processing are the 31 kDa chymotrypsin-like prostate specific antigen (Akiyama *et al.*, 1987; Christensson *et al.*, 1990) shown previously to be inhibited by DFP and PMSF and the hGK-1 gene product. The latter is an arginine-restricted trypsin-like protease of the human glandular kallikrein family, which is strongly suspected to be present in seminal plasma, since its messenger RNA is expressed at high levels in the prostatic epithelium (Chapdelaine *et al.*, 1988; Paradis *et al.*, 1989). However, there has been no report yet of its isolation from seminal plasma. On the other hand, some trypsin-like activity has been reported to contaminate purified preparations of PSA (Watt *et al.*, 1986; Lilja, 1985). This activity could be eliminated by affinity chromatography on an aprotinin or benzamidine column (Christensson *et al.*, 1990; Akiyama *et al.*, 1987). Moreover, a 36 kDa protease inhibited by benzamidine has been reported recently in prostatic fluid (Wilson *et al.*, 1993). The benzamidine-sensitive proteolytic activity observed in the present experiments might originate from that protease. As a working hypothesis we propose that PSA, shown previously to be inhibited by DFP and PMSF (Watt *et al.*, 1986; McGee & Herr, 1988), is the protease inhibited by PMSF in the present experiment, and that the hGK-1 gene product represents the other protease inhibited by benzamidine. However, definite identification of the proteases involved will require further experiments, including purification of the 52 kDa SPMI precursor and proteases.

The SPMI precursor degradation phenomenon observed in the present study closely resembles the process of seminal liquefaction and the simultaneous degradation of the predominant protein of seminal coagulum (Lilja & Laurell, 1985; Lilja *et al.*, 1987), as well as the processing and characteristics of a seminal vesicle-specific antigen (McGee & Herr, 1987, 1988). The vesicular origin of all these proteins, their molecular processing and the accompanying changes in molecular masses are strikingly similar. Amino acid composition and sequence information about the SPMI precursor and/or its proteolytic fragments should provide further information on possible relationships with these proteins.

The actual physiological role of SPMI remains speculative. Whether it plays an active and/or beneficial role during the reproductive process is presently unknown. The level of biological activity of the SPMI precursor in SVF and in liquefied seminal plasma is much higher than that required to arrest the motility of demembranated–reactivated spermatozoa. This demonstrates the effectiveness of the sperm plasma membrane in preventing the access of SPMI to the dynein ATPase on the axoneme (Iwamoto & Gagnon, 1988a). However, the high level of SPMI biolog-

ical activity present in SVF and in freshly ejaculated semen is within the range (600–1600 U/ml) shown to interfere with the motility of intact spermatozoa (Iwamoto & Gagnon, 1988b). Thus, rapid processing of the SPMI precursor and the associated decrease in biological activity by prostatic proteases, to a level at which sperm motility is not readily affected, becomes essential for proper sperm function. Whether some cases of male infertility (asthenozoospermia) could be attributed to improper, or lack of, processing of the SPMI precursor remains to be investigated.

Acknowledgments

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Chapter III

Sperm motility inhibitor from human seminal plasma: association with semen coagulum

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Preface

In Chapter II, we observed that SPMI is present in seminal vesicle fluid as a predominant 52 kDa precursor which is rapidly degraded in semen by proteases originating from the prostate. This phenomenon occurred, after ejaculation in parallel with semen liquefaction. The mass of the SPMI precursor and of its proteolytic products together with the fact that proteases originating from the prostate are responsible for its processing suggest that the SPMI precursor shares multiple similarities with the predominant proteins forming the structural components of semen coagulum. To investigate these similarities further, the isolation and analysis of the coagulated and soluble components of semen was undertaken. In the following chapter, we have determined the distribution of SPMI in these two semen components and evaluated whether processing of the SPMI precursor is associated with the phenomenon of semen coagulation and liquefaction.

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Sperm motility inhibitor from human seminal plasma: association with semen coagulum

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Human seminal plasma contains a sperm motility inhibitor (SPMI) originating from the seminal vesicles as a 52 kDa precursor form that is rapidly degraded by prostatic proteases after ejaculation. In this study, the distribution of SPMI biological activity and antigens was analysed in chemically induced, as well as naturally occurring, arrest of semen liquefaction. SPMI activity was detected exclusively in the coagulated semen fraction at 2200 ± 560 IU, whereas total seminal plasma proteins separated more evenly between soluble and coagulated components (91 ± 19 and 65 ± 18 mg, respectively). An SPMI antiserum recognized different forms of SPMI precursors at 52, 38, 35, 33 and 20 kDa in the coagulum while the soluble protein fraction contained only one major immunoreactive band at 15 kDa. High levels of SPMI activity (1500 ± 180 IU/ml) together with high molecular mass forms of SPMI precursor and low sperm motility (26%) were detected in semen samples that failed to liquefy spontaneously at room temperature. Addition of prostatic secretions to the non-liquefying samples caused a decrease of SPMI activity (330 ± 17 IU/ml) and transformed the SPMI precursor into low molecular mass forms (14–22 kDa) with a concomitant increase in sperm motility to 49%. The results suggest that SPMI is highly associated with the seminal coagulum components as very active forms that may adversely affect sperm motility when not properly processed after ejaculation.

Key words: protein precursor/proteolytic processing/prostate/semen/sperm motility

Introduction

At ejaculation, fluids from the various accessory glands of the male reproductive tract mix with the sperm rich fluid from the epididymis. Analysis of semen fractions collected by the split ejaculate method provided evidence that secretions of the various glands along the male reproductive tract are emitted sequentially (Tauber *et al.*, 1975, 1976). The prostatic secretions are emitted first together with the epididymal fluid, and are followed in the later stage of ejaculation by the secretions from the seminal vesicles (Lindholmer, 1973; Eliasson and Johnsen, 1986). Semen coagulates spontaneously (Amelar, 1962), and then liquefies through the action of proteolytic enzymes (Tauber *et al.*, 1976, 1980). The structural proteins of the seminal coagulum have been identified (Lilja and Laurell, 1985; Lilja *et al.*, 1987); these are constituted of a predominant 52 kDa protein, semenogelin which interacts with two less abundant 71 and 76 kDa related proteins by disulphide bonds (Lilja *et al.*, 1989). Non-covalent interactions between these proteins appear to be important for the coagulum structure since the coagulum can be solubilized in the denaturing agent guanidine hydrochloride (Lilja and Laurell, 1985). However, the exact molecular mechanism by which the coagulum is formed remains to be elucidated. On the other hand, it has been proposed that semen liquefaction occurs by proteolytic degradation of these proteins by the prostate-specific antigen (Lilja, 1985; Lilja *et al.*, 1987; McGee and Herr, 1987, 1988; Lee *et al.*, 1989).

The motility of mammalian spermatozoa can be arrested by non-ionic detergents and subsequently reinitiated by addition of magnesium.ATP (Lindemann and Gibbons, 1975; Mohri and Yanagimachi, 1980; de Lamirande *et al.*, 1983). However, the presence of seminal plasma can prevent the reinitiation of sperm motility in demembranated sperm models (de Lamirande *et al.*, 1983, 1984). A basic protein of 18–22 kDa has been purified from human seminal plasma and shown to contain the inhibitory activity. This protein was named the seminal plasma motility inhibitor (SPMI, Iwamoto and Gagnon, 1988a). There is good evidence suggesting that at least on demembranated reactivated spermatozoa, SPMI blocks the motility by interfering with the activity of the force generating ATPase of the axoneme dynein arms (Iwamoto and Gagnon, 1988a; Gagnon *et al.*, 1992; Iwamoto *et al.*, 1992). However, SPMI can also affect the motility of intact spermatozoa in a dose-dependent manner (Iwamoto and Gagnon, 1988b), by a yet unknown mechanism.

Luterman *et al.* (1991) have shown that the human SPMI originates exclusively from the seminal vesicles and that the specific activity in these glands' secretions is eight to nine times higher than that in seminal plasma. In a previous study (Robert and Gagnon, 1994), we provided evidence that this difference in specific activity is explained by the presence of a predominant 52 kDa SPMI precursor molecule in the seminal vesicles which has higher biological activity than the forms found in seminal plasma. After ejaculation the precursor is rapidly processed, along with its associated intermediate mass

(25–40 kDa) polypeptides, by proteases originating from the prostatic secretions. These proteases could be successfully inhibited by the addition of the protease inhibitors phenylmethylsulphonyl fluoride (PMSF) and benzamidine and by the heavy metal chelator 1,10-phenanthroline. The molecular processing of the SPMI precursor shows close similarities with that of the 52 kDa semenogelin, the main structural component of the seminal coagulum (Lilja, 1985; Lilja and Laurell, 1985; Lilja *et al.*, 1987). These observations led us to investigate whether SPMI precursor polypeptides could be associated with the components of semen coagulum. To do this, normal semen samples collected under chemical conditions that prevented normal semen liquefaction, as well as a naturally occurring case of non-liquefying semen were analysed.

Materials and methods

Materials

Glycine, HEPES, sodium dodecylsulphate (SDS) and dithiothreitol (DTT) were purchased from ICN Biomedicals (Montreal, Canada). Triton X-100 and 1,10-phenanthroline from Sigma Chemical Co. (St. Louis, MO, USA). Acrylamide, β-mercaptoethanol (β-ME), Coomassie Blue, Nitroblue tetrazolium chloride (NBT) and bromo-chloro-indolphosphate (BCIP) from Bio-Rad (Mississauga, Canada). Adenosine triphosphate (ATP) was from Boehringer Mannheim (Laval, Canada). Ultrapure urea was obtained from Bethesda Research Laboratories (Bethesda, MD, USA), Percoll from Pharmacia (Baie d'Urfé, Canada), porcine serum from Gibco BRL (Burlington, Canada) and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Ig) G from Jackson Immunoresearch Laboratories (West Grove, PA, USA). All other chemicals were of reagent grade at least.

Prostatic secretions collection

Prostate specimens were obtained at autopsy from victims of accidental death ($n = 2$) with a mean age of 41 ± 2 years. The prostate was dissected free from surrounding tissues and opened longitudinally through the urethra. Prostatic secretions were collected after thorough washing of the gland with 0.9% NaCl solution, by firmly squeezing the gland, and collecting fluid at the level of the verumontanum. The secretions were then diluted in an equal volume of HEPES saline solution (25 mM HEPES, 100 mM NaCl) at pH 8.0, mixed and centrifuged for 20 min at 10 000 *g*. The supernatants were collected and frozen at –70°C until used.

Isolation of coagulated and soluble semen fractions

Semen was provided by healthy male volunteers by masturbation, directly into sterile containers containing 0.5 ml of a 0.5 M solution of 1,10-phenanthroline. The sample was mixed and stored immediately in ice-cold water. This procedure ensured a minimal final concentration of 50 mM 1,10-phenanthroline for an average ejaculate ≤5 ml and prevented semen liquefaction (Lilja and Laurell, 1985). In a 15 ml conical tube, 10 ml of HEPES saline solution was added to coagulated semen. The mixture was stirred extensively to extract a maximum of soluble components and centrifuged at 1000 *g* for 10 min. The supernatant was removed and kept on ice. An additional 10 ml of HEPES saline solution was added to the pellet of insoluble components, the content mixed and centrifuged again. This procedure was repeated four times. All supernatants were pooled and centrifuged again for 15 min at 10 000 *g*. Finally the washed coagulum was solubilized in 8 M urea/HEPES saline solution. The presence of urea was shown to have no effect on SPMI biological activity assay. For comparison, some semen samples were also allowed to liquefy spontaneously.

Analysis of a naturally occurring case of non-liquefying semen

Semen obtained from a patient consulting the Fertility Centre of the Royal Victoria Hospital (Montreal, Canada) for routine semen analysis was observed to remain in a highly coagulated form *in vitro*, even after 2 h at room temperature. This period greatly exceeds the normal time required for full semen liquefaction, usually 5–20 min. Three different such non-liquefying semen samples were analysed for the presence of SPMI following incubation with prostatic secretions or buffer (3:1, v/v) for 2 h at room temperature. The SPMI biological activity was determined after complete semen solubilization in 8 M urea/HEPES saline solution and proteins were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Sperm motility was measured using the CellSoft computer-assisted digital analysis system (Cryo Resources, Montgomery, NY, USA).

SPMI biological activity assay

Percoll washed human spermatozoa were demembranated in a medium containing 0.1% Triton X-100, 0.2 M sucrose, 0.025 M potassium glutamate pH 8.0, 0.035 M Tris-HCl pH 8.0 and 1 mM DTT (de Lamirande *et al.*, 1983). After demembranation, the motility of spermatozoa was reinitiated by the addition of 0.5 mM magnesium.ATP. One inhibitory unit (IU) of SPMI biological activity is defined as the minimal amount of proteins that completely inhibits the motility of demembranated-reactivated spermatozoa within 5–10 s in 1 ml of demembranation-reactivation medium.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins from seminal plasma and other fluids were separated by SDS-PAGE according to Laemmli (1970) using the Bio-Rad, Mini Protean II electrophoresis system, after solubilization in SDS-sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercapto-ethanol, 0.0125% Bromophenol Blue). The stacking and running gels contained 5 and 15% acrylamide respectively. Electrophoresis was carried out at a constant current of 20 mA for 1 h. The gels were then stained with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol/10% acetic acid and destained successively in 40% methanol/10% acetic acid and 10% methanol/10% acetic acid.

Immunoblotting and immunodetection

Following electrophoresis, proteins were electrotransferred from slab gels onto Nitroplus nitrocellulose membrane (Micron Separations Inc., Westboro, MA, USA) for 2 h at 70 V. The transfer buffer was composed of 25 mM glycine, 25 mM ethanolamine, 0.01% SDS and 20% methanol at pH 9.8. After transfer, the membranes were air dried and stored at 4°C until used. Membranes were then first stained in 0.2% Ponceau S in 3% acetic acid to visualize proteins and molecular weight markers. After destaining in 10 mM Tris-HCl, 0.9% NaCl (TBS) pH 7.4, non-specific sites on the membranes were blocked with 10% swine serum in TBS containing 0.25% Tween-20 and 0.02% sodium azide (blocking solution) for 1 h at room temperature. The membranes were then incubated for 1 h with a rabbit anti-human SPMI antiserum generated against a 19 kDa SPMI form purified from human seminal plasma (Luterman *et al.*, 1991) diluted 1:200 in blocking solution. After four successive 10 min washes in TBS containing 0.25% Tween-20, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1:2000 in blocking solution. Membranes were then washed four times in TBS containing 0.25%

Table I. Distribution of sperm motility inhibitor (SPMI) biological activity and total proteins between the coagulated and soluble semen components. Results are expressed as mean ± SEM ($n = 4$)

	Coagulum	Soluble fraction	Liquefied semen
Biological activity (IU)	2200 ± 560	ND	470 ± 60
Total protein (mg)	91 ± 19	65 ± 18	120 ± 2
Specific activity (IU/mg)	23 ± 2	-	4 ± 0.5

ND = no activity detected.

Tween-20. Antigens were visualized by incubation in alkaline phosphatase buffer (100 mM Tris-HCl, 1 mM $MgCl_2$, pH 9.5) containing the substrates NBT and BCIP at 0.1 and 0.05 mg/ml respectively. The reaction was terminated by washing the membrane in distilled water.

Protein determination

The concentration of proteins in the various samples was measured with the bicinchoninic acid (Pierce Chemical Co., Rockford, IL, USA) assay using the procedure described by Smith *et al.* (1985). Bovine serum albumin was used as a standard.

Results

Analysis of SPMI biological activity and antigens in coagulated semen

Following ejaculation into a solution of 1,10-phenanthroline, semen samples were separated into soluble and coagulated components as described above. SPMI biological activity was exclusively detected in the coagulated components with a total of 2200 ± 560 IU/ejaculate (mean volume of 3 ml; Table I). No SPMI activity was detected in the supernatant. The total SPMI biological activity in normally liquefied semen was nearly 5-fold lower (470 ± 60 IU) than that of the coagulated components. On the other hand, total semen proteins distributed more evenly between the coagulated and the soluble components of non-liquefied semen with 91 ± 19 and 65 ± 18 mg of proteins, respectively. The total amount of proteins in normally liquefied semen amounted to 120 ± 2 mg, 4 h after ejaculation. SPMI specific activity was 6-fold higher in the coagulated fraction with 23 ± 2 IU/mg compared with 4 ± 0.5 IU/mg in liquefied semen.

Major differences were observed in the protein composition of coagulated and soluble components of semen when analysed by SDS-PAGE (Figure 1A). Coagulated components (lane 2) differed mainly from soluble components (lane 3) by the presence of intensely stained bands at 52, 38, 35, 33, 20 and 18 kDa. In contrast, the protein composition of the soluble components did not contain intensely stained bands at such molecular masses but were rather characterized by the presence of 90 and 66 kDa bands, a doublet around 48–49 kDa and fainter bands of smaller molecular mass 14–21 kDa. The electrophoretic profile of liquefied semen (lane 1) also differed from that of the coagulum, but closely resembled that of the soluble components. Immunodetection of SPMI antigens in the same samples using SPMI antibodies revealed the presence of immunoreactive proteins at 52, 38, 35, 33 and 20 kDa, in coagulated components (Figure 1B, lane 2). These protein

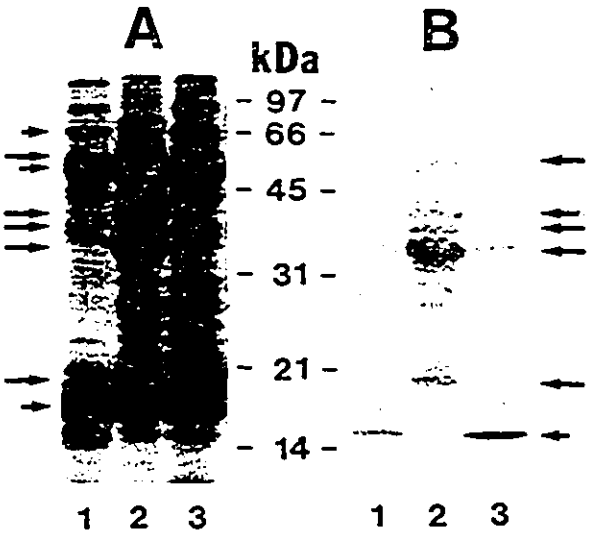

Figure 1. Electrophoretic profile and distribution of sperm motility inhibitor (SPMI) antigens in coagulated and soluble semen components. Semen collected into a solution of 1,10-phenanthroline was processed as described above. An aliquot of the separated semen components (30 µg) was mixed with sodium dodecyl sulphate (SDS) sample buffer and heated to 95°C for 3 min before analysis by SDS-polyacrylamide gel electrophoresis (A) and immunoblotting with SPMI antiserum (B). Normally liquefied semen (1), coagulated components (2) and soluble components (3). Arrows identify specific bands described in the text: long arrows relate to lane 2, short arrows to lanes 1 and 3.

bands corresponded to the major Coomassie Blue stained bands described above for this fraction. However, one intensely stained protein at 18 kDa did not show any immunoreactivity. In the soluble components (lane 3), immunoreactivity was observed at 15 kDa and faintly at 33 kDa. In liquefied semen, the same 15 kDa polypeptide was also observed and was the only immunoreactive band detected. However, it was absent from the coagulated semen components. On the other hand, the 33 kDa band, present in the soluble components, seemed to correspond to the 33 kDa protein observed in the coagulated components. When the same samples were probed with pre-immune serum, no immunoreactive band was detected (not shown).

Analysis of SPMI biological activity, antigens and sperm motility in naturally occurring, non-liquefying semen samples

SPMI biological activity as measured in highly coagulated but non-treated semen samples that failed to liquefy spontaneously was 1500 ± 180 IU/ml immediately after ejaculation (Table II). The corresponding sperm motility was 26 ± 3%. The SPMI biological activity and sperm motility level in semen remained almost unchanged at room temperature, after 2 h (1300 ± 205 IU/ml and 21 ± 1%). However, when the non-liquefying semen sample was incubated with exogenous prostatic secretions (3:1, v/v), SPMI biological activity decreased to 330 ± 17 IU/ml in parallel with the disappearance of the coagulated material and an increase in sperm motility to 49 ± 5% over the 2 h period. SPMI specific activity went from 20 ± 4 and 19 ± 5 IU/mg in the sample to which buffer

Table II. Seminal plasma motility inhibitor (SPMI) biological activity and associated sperm motility in non-liquefying semen. Results are expressed as mean ± SEM (n = 3)

	Semen + buffer After 0 min	Semen + buffer After 2 h	Semen + prostate fluid After 2 h
Biological activity (IU/ml)	1500 ± 180	1300 ± 205	330 ± 17
Specific activity (IU/mg)	20 ± 4	19 ± 5	6 ± 1
Sperm motility (%)	26 ± 3	21 ± 1	49 ± 5

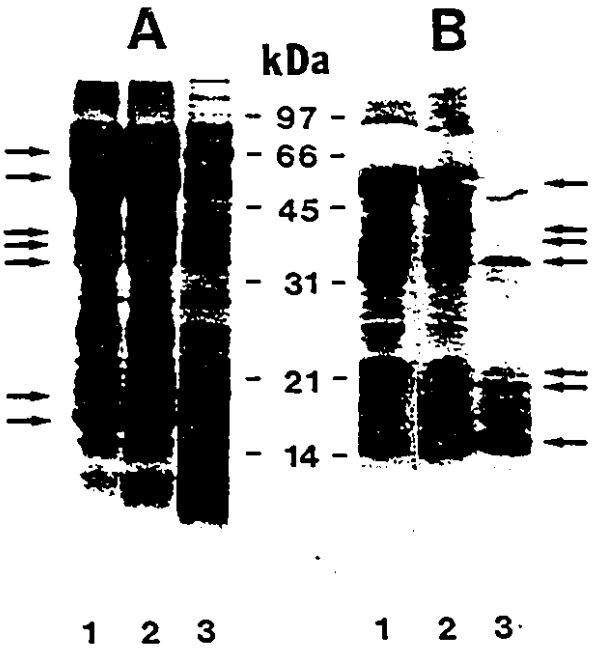

Figure 2. Electrophoretic profile and immunodetection of sperm motility inhibitor (SPMI) antigens in non-liquefying semen. An aliquot of the whole semen (30 µg) was mixed with sodium dodecyl sulphate (SDS) sample buffer and heated to 95°C for 3 min before analysis by SDS-polyacrylamide gel electrophoresis (A) and immunoblotting with SPMI antiserum (B). Non-liquefying semen, immediately after ejaculation (1), non-liquefying semen following 2 h incubation at room temperature with HEPES saline solution buffer (2), or prostatic secretions (3:1, v/v) (3). Arrows identify specific bands described in the text.

was added, immediately and 2 h after ejaculation respectively, to 6 ± 1 IU/mg in the sample treated with prostatic secretions for 2 h.

The change in SPMI specific activity and sperm motility, following treatment with prostatic secretions, was accompanied by changes in the proteins' electrophoretic profile (Figure 2A). Non-liquefying semen contained predominant proteins at 66, 52, 38, 33, 20 and 18 kDa which showed intense immunoreactivity when probed with SPMI antibody on the immunoblots (Figure 2B, lane 1). In the sample treated with prostatic secretions, the high molecular mass bands at 52, 38 and 35 kDa had disappeared after 2 h (lane 3). Only small amounts of 49 and 33 kDa polypeptides, as well as most bands <22 kDa remained. On the other hand, almost no change occured in the electrophoretic profile of the non-liquefying semen after 2 h of incubation with buffer (lane 2). Only two bands at 22 and 20 kDa decreased in intensity.

Discussion

The results of the present study clearly demonstrate that SPMI biological activity and antigenicity are tightly associated, and seem to aggregate with the non-soluble components of non-liquefied semen, or semen coagulum. No activity was detected in the soluble fraction. Under the present experimental conditions and considering the sensitivity of the assay, a level equivalent to 5% of the SPMI activity in whole semen would have easily been detected. Thus, in non-liquefying semen, the great majority (> 95%) of the SPMI biologically active and immunoreactive polypeptides exist as coagulated forms, or associated with the semen coagulum. The specific activity of SPMI was found to be six-fold higher in the coagulum than in liquefied seminal plasma. This is explained in part by the presence in the coagulum of very active SPMI precursor forms originating from the seminal vesicles (Robert and Gagnon, 1994) as well as the SPMI enrichment obtained when the soluble proteins are eliminated by washing coagulated semen. To isolate the coagulum components from the soluble ones in semen, we elected to use the heavy metal chelator 1,10-phenanthroline in the present experiments, since it was previously shown to inhibit the liquefaction of coagulated semen (Lilja and Weiber, 1984; Lilja and Laurell, 1985). The exact mechanism of this inhibition is still ill-defined but likely involves the inhibition of zinc-dependent seminal proteases. We have also recently reported that proteolytic degradation of the SPMI precursor by prostatic secretions can be partially inhibited by 1,10-phenanthroline (Robert and Gagnon, 1994).

Immunoreactive polypeptides of various sizes (20–52 kDa) were always detected in all coagula analysed. On the other hand, the staining intensity ratio of these polypeptides in the coagulated components varied from one sample to another. The 52 kDa immunoreactive protein which was previously identified as the predominant precursor form of SPMI (Robert and Gagnon, 1994) was always associated, in the coagulum, with other polypeptides at 38, 35, 33 and 20 kDa. The presence of these polypeptides in the coagulated components probably reflects partial degradation of the 52 kDa SPMI protein which already took place in spite of the use of 1,10-phenanthroline to prevent its degradation and semen liquefaction. A similar observation was also reported previously (Lilja and Laurell, 1985). This partial cleavage of the 52 kDa SPMI precursor is possibly the consequence of the time delay before 1,10-phenanthroline completely chelates the metal ion required for normal precursor degradation. The heterogeneity in sizes of SPMI molecules in the coagulum could also be an inherent characteristic of these seminal vesicle proteins. We have often observed the presence of multiple immunoreactive SPMI forms

in freshly collected seminal vesicle fluid (data not shown). It is possible that SPMI precursors are secreted under various mass forms directly from the seminal vesicle epithelium, due to intracellular protein processing prior to secretion, or to alternative mRNA splicing. It may also be the result of partial proteolysis occurring in the lumen of the seminal vesicles.

The soluble components, obtained from multiple washings of the coagulated semen, do not simply represent a non-specific, partial solubilization of coagulum proteins since their electrophoretic pattern considerably differed from that of the coagulated material. They rather likely represent a set of distinct soluble proteins having no active role to play in the coagulum network, such as albumin, lactoferrin, transferrin and other proteins contributed by blood plasma (Tauber *et al.*, 1975) or by the secretions originating from the various male accessory glands. It is not possible to conclude from the results of the present experiments whether SPMI is an actual contributing structural component of the seminal coagulum structure. Nonetheless, it appears to be tightly associated with the coagulated components.

The presence in high concentrations of stable and highly active (1500 ± 180 IU/ml) SPMI precursor forms in a semen that fails to spontaneously liquefy, greatly contrasts with that of normal semen which liquefies within 20 min after ejaculation and whose SPMI biological activity drops to levels of 150–250 IU/ml within 2 h (Robert and Gagnon, 1994). We reported that serine and/or metallo-proteases, originating from the prostate, were responsible for SPMI precursor degradation. The present results thus seem to indicate that the quantity of prostatic secretions and/or their proteolytic activity content is inadequate in this particular semen sample. This conclusion is supported by the observation that addition of exogenous prostatic secretions triggered the normal processing of SPMI precursor into less active and lower mass forms of SPMI in parallel with semen liquefaction. The absence of normal proteolytic activity in the non-liquefying semen could be the consequence of a decrease in the secretory activity of the prostate, obstruction of the excretory path or alternatively of a reduced level of proteolytic activity in those secretions. The presence of additional immunoreactive bands, in the semen sample liquefied with prostatic secretions (Figure 2B, lane 3), that are not observed in normally liquefied semen (Figure 1B, lane 1) is likely the consequence of incomplete degradation of SPMI precursor in the former. The use of two-fold diluted and frozen–thawed prostatic secretions, which have lower proteolytic activity than normal whole semen, may explain this difference.

It is well established that sperm motility is higher in the initial fraction of the ejaculate, which is composed mostly of prostatic secretions and spermatozoa, than in the final fraction, enriched in seminal vesicle secretions (Lindholmer, 1973). A preliminary study demonstrated that SPMI activity level and its stability with time following ejaculation were higher in the final fraction of the ejaculate (unpublished results). This finding thus suggests a possible role of SPMI in that phenomenon.

The results clearly demonstrate that semen liquefaction is associated with the degradation of the SPMI precursor forms present in coagulated semen and an accompanying four-fold reduction in specific activity. However, the exact mechanism responsible for the difference in sperm motility inhibitory activity between the high mass SPMI and the degraded forms is still unclear. It may be related to the loss of a specific domain or to structural differences between the precursor and degraded forms which alter the affinity of SPMI for its target site on spermatozoa.

Ejaculated spermatozoa are immotile immediately after ejaculation, being entrapped in the semen coagulum, and only gain progressive motility as semen liquefies (Amelar, 1962). The results of the present study suggest an explanation for this observation. SPMI was shown to exist as precursor forms in coagulated semen. The high level of SPMI activity associated with these precursor forms appears to adversely affect the motility of intact spermatozoa, as suggested by the poor motility of spermatozoa in the non-liquefying semen sample case. Moreover, when SPMI activity was reduced considerably by prostatic secretions, with concomitant SPMI degradation, there was a clear increase in sperm motility up to 49%. These results suggest a possible clinical use of proteases to process non-liquefying semen samples from which the recovery of motile spermatozoa is poor following standard sperm preparation methods. In theory, addition of various proteases (i.e. trypsin, chymotrypsin, etc.) could induce the degradation of the high molecular mass SPMI molecules, and induce semen liquefaction and the release of motile spermatozoa. However, the use of proteases which are naturally occurring in semen and are well characterized, such as prostate-specific antigen, would probably be safer and more physiological. Ideally, when such enzymes become available in a safe, recombinant and pathogen-free form, they should be useful to treat non-liquefying semen samples. The use of an endogenous protease might avoid the hydrolysis of other important seminal or sperm proteins and eliminate the associated detrimental effects on the success of assisted reproduction that less 'physiological' proteases might have.

From the data currently available, the properties of the 52 kDa SPMI precursor and the 52 kDa semenogelin demonstrate striking similarities. Both proteins are associated with semen coagulum and are degraded concomitantly during semen liquefaction producing multiple polypeptide intermediates of similar masses (Lilja, 1985; Lilja and Laurell, 1985; Lilja *et al.*, 1987). Purification of the 52 kDa SPMI precursor and analysis of its amino acid sequence will be required before a definitive conclusion can be reached on whether these two proteins are identical.

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Chapter IV

Purification and characterization of the active precursor of a human sperm motility inhibitor secreted by the seminal vesicles

Martin Robert and Claude Gagnon

Preface

In the previous chapter, evidence that SPMI precursor is associated with the proteins of coagulated semen was provided. Its association with the low sperm motility found in coagulated semen was suggested from the study of an infertile patient whose semen does not liquefy spontaneously. SPMI precursor may thus be an important factor contributing to some cases of male-related infertility. In order to get more definite answers about the molecular nature of SPMI precursor, its purification was undertaken. The results presented in the following chapter describe: a novel method for the purification of SPMI precursor, the characterization of its activity on intact human spermatozoa, and provide details about its molecular nature.

Purification and characterization of the active precursor of a human sperm motility inhibitor secreted by the seminal vesicles.

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SUMMARY

Human seminal plasma contains a sperm motility inhibitor originating from seminal vesicles as a precursor form which is degraded into smaller peptides by prostatic proteases shortly after ejaculation. The seminal plasma sperm motility inhibitor (SPMI) precursor was purified by a combination of cation-exchange chromatography on S-Sepharose followed by C_4 reverse-phase high performance liquid chromatography (RP-HPLC) directly from seminal vesicle fluid or washed seminal coagulum. The purification procedure yielded a protein of apparent homogeneity, with a molecular mass of 52 kDa by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). It migrated as a 105 kDa protein by molecular sieving under denaturing conditions. The purified SPMI precursor could be digested by the prostatic protease prostate-specific antigen (PSA), causing a 76 ± 4% drop in biological activity and transformation into low molecular mass SPMI polypeptides (5-20 kDa), similar to those observed in liquefied semen. The N-terminal amino acid sequences of three degradation peptides were obtained by Edman degradation and found to correspond to residues 45-50, 85-90, and 137-143 of semenogelin, a protein characterized as the major structural component of semen coagulum. The amino acid composition of SPMI precursor was found to be almost identical to that of semenogelin. Moreover, the mass of the precursor was estimated at 49, 677 Da by electrospray-ionisation mass spectrometry, a value in close agreement with the expected mass of semenogelin according to its cDNA sequence. SPMI precursor was found to inhibit progressively sperm motility in a dose-dependent manner, with complete immobilization at 500 U/ml of SPMI. The motility of completely immobilized spermatozoa could be partially recovered following washing of the cells. The results suggest that SPMI precursor is the major component of the seminal vesicles secretions and seminal coagulum, and that it may have significant effects on sperm motility in freshly ejaculated semen. It can be degraded by PSA in a manner reminiscent to its processing in whole semen. All characteristics of SPMI precursor suggest that it is identical to semenogelin.

INTRODUCTION

Human seminal plasma is a complex biological fluid formed from the mixing of various fluids of the male reproductive tract, namely the secretions of the testes, epididymides, seminal vesicles, prostate and Cowper's glands (Mann and Lutwak-Mann, 1981). Immediately following ejaculation, human semen spontaneously coagulates into a semi-solid gelatinous mass which then liquefies within 5 to 20 minutes (Amelar, 1962; Tauber et al., 1976). Semen coagulum appears to be mainly constituted of a predominant 52 kDa protein, known as semenogelin, and two less abundant 71 and 76 kDa semenogelin-related proteins (Lilja and Laurell, 1985; Lilja et al., 1987; 1989). While some hypotheses have been proposed (Polak and Daunter, 1989; Lilja et al., 1987), the mechanism by which human semen coagulum components coagulate has not yet been elucidated. On the other hand, semen liquefaction is believed to occur following proteolytic degradation of these proteins by the prostatic enzyme prostate-specific antigen (PSA) (Lilja et al., 1987; Lilja, 1985; McGee and Herr, 1988). This enzyme, widely used as a marker of prostatic malignancies (Oesterling, 1991), was originally purified from seminal plasma as a semen specific protein (Wang et al. 1979). PSA is a 33 kDa protein which belongs to the kallikrein family of proteases (Watt et al., 1986; Schaller et al., 1987; Lundwall and Lilja, 1987) and is the most abundant protease secreted in seminal plasma, with physiological concentrations ranging between 0.2 and 5 mg/ml (Wang et al., 1979; Oesterling, 1991).

In the epididymis, spermatozoa are maintained in an immotile state (Eddy and O'Brien, 1994). Progressive sperm motility is initiated after ejaculation when epididymal spermatozoa come into contact with components of seminal plasma (Lindholmer, 1974). While prostatic secretions can stimulate sperm motility, the secretions of seminal vesicles have long been

known to be detrimental to sperm motility (Lindholmer, 1973). However, the specific components responsible for these detrimental effects have not yet been well characterized. Factors that can inhibit sperm motility have been reported in the seminal plasma and seminal vesicle fluid of various mammals including boar (Strzezek et al., 1992; Iwamoto et al., 1992; Jeng et al., 1993), bull (Al-Somai et al., 1994) and mouse (Peitz, 1988). In humans, Iwamoto and Gagnon (1988a) reported the purification of a basic seminal plasma protein of 19 kDa that can inhibit the motility of Triton X-100 demembranated spermatozoa reactivated by the addition of Mg.ATP. Under these conditions, it appears to act by interfering with dynein ATPase, the key enzyme responsible for flagellar motility (Iwamoto and Gagnon, 1988a). This factor, named "seminal plasma motility inhibitor" (SPMI) was subsequently shown to inhibit the motility of intact spermatozoa (Iwamoto and Gagnon, 1988b) through a mechanism not yet characterized.

Human SPMI originates exclusively from the seminal vesicles, as a potent precursor form of 52 kDa (Luterman et al., 1991; Robert and Gagnon, 1994). This precursor is rapidly degraded and partially inactivated by prostatic serine and/or metallo-proteases immediately after ejaculation, in parallel with semen liquefaction. This degradation transforms the SPMI precursor into less active, smaller molecular mass forms of 17-21 kDa found in liquefied semen that are compatible with sperm motility. SPMI precursor antigens and a potent sperm motility inhibitory activity are associated with the coagulated components of non-liquefied seminal plasma, and experimental evidence is suggesting that improper SPMI precursor processing after ejaculation may result in poor sperm motility and reduced fertility (Robert and Gagnon, 1995). These results also demonstrate that SPMI shares multiple characteristics with semenogelin.

The properties of SPMI precursor indicate that it may be responsible for the temporary immobilization of spermatozoa in coagulated semen. Proper processing of SPMI precursor during semen liquefaction appears essential for spermatozoa to acquire progressive motility. In the present study, we developed the first purification procedure for SPMI precursor and characterized some of its properties. The protein displays potent, reversible sperm immobilizing activity and appears to form macromolecular aggregates. We have also examined the capacity of the enzyme PSA to hydrolyze SPMI precursor and analyzed some of its fragments. The results provide strong evidence that SPMI precursor is the predominant protein in seminal coagulum and seminal vesicle fluid, and that it is identical to semenogelin.

MATERIALS AND METHODS

Materials

Glycine, N-2-hydroxyethyl piperazine N-2'-ethanesulfonic acid (HEPES), cyclohexylaminopropane sulfonic acid (CAPS), sodium dodecylsulfate (SDS), dithiothreitol (DTT) were purchased from ICN Biomedicals (Montréal, QC, Canada); acrylamide, β-mercaptoethanol (β-ME), Coomassie Blue, nitroblue tetrazolium chloride (NBT) and bromo-chloro-indolphosphate (BCIP) from Bio-Rad (Mississauga, ON, Canada). Iodoacetamide was from Sigma Chemical Co. (St-Louis, MO) and adenosine triphosphate (ATP) from Boehringer Mannheim (Laval, QC, Canada). Ultrapure urea was obtained from Bethesda Research Laboratories (Bethesda, MD), HPLC-grade trifluoroacetic acid (TFA) and acetonitrile from J.T. Baker (Toronto, ON, Canada), S-Sepharose Fast Flow and Percoll from Pharmacia (Baie d'Urfé, Canada), porcine serum from Gibco-BRL (Burlington, ON, Canada) and alkaline phosphatase-conjugated goat anti-rabbit and anti-mouse IgG from Jackson Immunoresearch Laboratories (West Grove, PA). PSA purified from human seminal plasma was generously provided by Dr. J.Y. Dubé, Laval University (Québec, QC, Canada) or obtained commercially from Scripps Laboratories (San Diego, CA). All other chemicals were at least of reagent grade.

Seminal vesicle fluid collection

Tissue specimens were collected from patients undergoing radical prostatectomy. Seminal vesicle fluid (SVF) was collected by direct needle aspiration of the ligated gland. The fluid was diluted in an equal volume of HEPES saline solution (HSS, 25 mM HEPES, 100 mM NaCl, pH 8.0) containing 8 M urea to achieve complete solubilization of the viscous and

gelatinous components.

Isolation of seminal coagulum.

Semen from vasectomized volunteers was collected by masturbation into a sterile container to which 40 ml of ice-cold 50 mM sodium acetate (pH 4.5) were added immediately after ejaculation. The coagulum was then isolated by successive washings, according to a previously described method (Mandal and Bhattacharyya, 1992). The resulting coagulum was solubilized in an equal volume of 8 M urea/HSS and kept at 4°C until use.

Protein disulfide reduction and blocking.

Prior to chromatography, urea solubilized seminal vesicle fluid or seminal coagulum proteins were reduced by the addition of crystalline DTT to a final concentration of 25 mM and incubation at room temperature for 30 minutes. Reduced disulfides were then covalently blocked by addition of crystalline iodoacetamide to 125 mM and incubated at room temperature for another 30 minutes. The proteins were then precipitated in 94% ethanol at -70° C, resuspended in 8 M urea/HSS, and stored at the same temperature until use.

Isolation of motile spermatozoa.

Semen was provided by healthy volunteers by masturbation into sterile containers, after 3 days of sexual abstinence. After liquefaction, the semen was layered on a discontinuous Percoll density gradient made of 2 ml each of 20, 40, 65% Percoll and 0.2 ml of 95% Percoll buffered in HBS (25 mM HEPES, 130 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$ and 14 mM fructose, pH 8.0). Following centrifugation at 1300 x g for 30 minutes, highly motile and

morphologically normal spermatozoa from the 65-95% Percoll interface and the 95% Percoll layer were recovered and combined.

SPMI activity assay

Percoll washed human spermatozoa were demembranated in a medium containing 0.1% Triton X-100, 0.2 M sucrose, 0.025 M potassium glutamate, 0.035 M Tris-HCl pH 8.0 and 1 mM DTT and the motility was reinitiated by the addition of 0.5 mM Mg.ATP. Aliquots of the test solutions were then added to the assay medium containing the reactivated spermatozoa and observed under the microscope for inhibition of motility. One inhibitory unit of SPMI activity was defined as the minimal amount of proteins that completely inhibits, within 5 to 10 seconds, the motility of spermatozoa demembranated and reactivated in 1 ml of medium.

Sperm motility analysis

Sperm motility parameters were analyzed using the CellSoft computer-assisted semen analysis system (Cryo Resources Ltd., New York, NY). Five microliters of the sample to be analyzed were placed in a Makler counting chamber (10 µm depth) kept at room temperature. The chamber was placed under an Olympus BH-2 phase contrast microscope. Using a 50 X objective, the image was relayed to a television monitor via a video camera mounted on the microscope. The imaged was digitized and fed into a computer. The digital video image was then analyzed by the CellSoft program. The following user-defined settings were used: 20 frames to analyze at 30 frames per second; *minimum* samplings for motility and velocity were 3 and 10, respectively; minimum and maximum velocities were 10 and 250 µm per second;

pixel scale of 0.92 and cell size range of 5-25 pixels. Measurements were performed in at least five random fields and a minimum of 200 cells were analyzed for each sample. Both the percentage of motility and the sperm velocity (total length travelled divided by the total time the cell was tracked) were measured.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Proteins were separated by SDS-PAGE as previously described (Robert and Gagnon, 1994) and electrotransferred onto Nitroplus nitrocellulose membrane (Micron Separations Inc., Westboro, MA) for 1 hour at 70 V under alkaline conditions (10 mM CAPS pH 11.0, 0.01% SDS and 15% methanol). Immunodetection was performed, as previously described (Robert and Gagnon, 1995), using a rabbit anti-human SPMI antiserum generated against a 19 kDa SPMI form purified from human seminal plasma. For microsequencing, proteins were similarly transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membranes were washed extensively in water and polypeptide bands were carefully excised with a clean scalpel blade.

Amino acid analysis

Selected purified samples of SPMI precursor were subjected to 6N HCl vapor hydrolysis, for 18 hours at 110°C using a Pico-Tag workstation (Waters Associates, Bedford, MA). Samples were analyzed for their amino acid composition using a Beckman model 6300A autoanalyzer.

N-terminal amino acid sequencing

The amino acid sequencing was performed at the Protein Sequencing Facility of the Sheldon Biotechnology Center, McGill University. Individual polypeptide bands excised from Immobilon-P membranes were inserted into the sample cartridge. The N-terminal amino acid sequence was determined by Edman degradation using a Porton Instruments automated gas-phase sequenator.

Atmospheric pressure ionization mass spectrometry (API-MS)

Mass spectra were obtained in the positive mode on a triple stage mass spectrometer Model API-III (SCIEX, Toronto, ON, Canada). The samples were dissolved in 10% acetic acid and infused through a stainless steel capillary (100 µm I.D.) at a flow rate of 1µl/min. A stream of air (pneumatic nebulization) was introduced to assist in the formation of submicron droplets. These droplets were evaporated at the interface by nitrogen gas producing highly charged ions which were detected by the analyzer. The system calibration was performed with the ammonium adduct ions of polypropylene glycol of known mass to charge ratios throughout the detection range of the instrument (0-2470 atomic mass units). Instrument tuning, data acquisition and processing were controlled by a computer system. Simple algorithms correlated the charges produced by the polypeptides to their molecular mass.

Protein concentration determination

The concentration of proteins in all samples was measured with the bicinchoninic acid (Pierce Chemical Co., Rockford, IL) assay following the procedure described by Smith et al. (1985) using bovine serum albumin as a standard.

RESULTS

Purification of the SPMI precursor

The seminal vesicle fluid loaded on the S-Sepharose column contains multiple polypeptides spread over a wide range of molecular masses (Fig. 1C). Among these, the predominant 52 kDa precursor as well as multiple polypeptides (< 52 kDa) were recognized by SPMI antibodies. When applied on the cation exchanger, the majority of seminal vesicle fluid proteins did not bind to the resin and eluted in the column flow through (Fig. 1A). This peak of unretained proteins contained no SPMI activity, and the various polypeptides showed no immunoreactivity with SPMI antiserum (Fig 1C). Most bound proteins eluted as a single broad peak at NaCl concentrations between 40 and 200 mM, and all SPMI activity co-eluted in this peak of proteins. The early eluting polypeptides (40-100 mM NaCl) contained predominantly two 71 and 76 kDa proteins which were weakly immunoreactive to SPMI antiserum and little of the 52 kDa protein. On the other hand, the late eluting components (100-200mM NaCl) contained predominantly the 52 kDa protein together with other less abundant polypeptides of lower mass as well as small amounts of the 71 and 76 kDa proteins. Most of the polypeptides eluting at this position (15-50 kDa) were immunologically related to SPMI and likely represented degradation products of the precursor present in seminal vesicle fluid prior to purification (Robert and Gagnon, 1994). Only the late eluting fractions of that peak, which contained minimal amounts of the 71 and 76 kDa polypeptides were pooled owing to the difficulty in eliminating these high molecular mass polypeptides in the subsequent step. This initial purification step, resulted in a 3.9-fold enrichment of the 52 kDa SPMI precursor with an overall recovery of 75% of the initial SPMI activity (Table I).

Pooled S-Sepharose fractions were loaded on a C_4 RP-HPLC column equilibrated in 0.1%

TFA. Linear gradient elution (25–40% solvent B) gave rise to two major distinct protein peaks (Fig. 1B) eluting at 32 and 34% of solvent B, respectively. SPMI activity was present in both peaks. Subsequent analysis of the contents of each peak by SDS-PAGE and immunoblotting revealed that the first eluting peak was composed of multiple polypeptides of 15-50 kDa, the majority of which were reactive with SPMI antiserum (Fig. 1C). The second elution peak contained the 52 kDa SPMI precursor in homogeneous form. This second step yielded an overall 5-fold purification factor with a total recovery of 12% of the initial SPMI activity (Table I).

When washed semen coagulum solubilized in urea was used as the source of SPMI precursor, the majority of the proteins bound to the S-Sepharose resin (Fig. 2A). The chromatographic behavior of the eluting components was very similar to that observed with seminal vesicle fluid. However, the proteins in the flow through of the cation-exchanger column represented a smaller proportion of total proteins when compared with the procedure using seminal vesicle fluid. The pooled S-Sepharose fractions gave rise to similar elution peaks on C_4 RP-HPLC, and most of the activity was found in the second peak containing the 52 kDa polypeptide (Fig. 2B). The purification factor reached 2.2 when semen coagulum was used as a source of SPMI precursor and the overall recovery of SPMI activity was 18% (Table II).

Characterization of the SPMI precursor.

Gel filtration

When gel filtration chromatography was performed under denaturing conditions (8 M urea in HSS), the 52 kDa precursor eluted at an intermediate volume between gamma-globulin and bovine serum albumin (Fig. 3) at an estimated molecular mass of 105 kDa. However, under

native conditions (HSS alone) the purified SPMI precursor did not elute from the column, independently of the presence or absence of 0.5 M NaCl in the elution buffer. On the other hand, the presence of 0.1% Triton X-100 in the HSS eluant resulted in essentially the same profile as with urea (data not shown).

Effect of PSA on SPMI precursor.

Previous results have shown that SPMI precursor is processed in semen by a serine protease originating from the prostate (Robert and Gagnon, 1994). Since PSA is the most abundant protease in semen and its characteristics (Watt et al., 1986; Schaller et al., 1987) correspond well with previous observations on SPMI processing (Robert and Gagnon, 1994), we investigated whether the purified SPMI precursor could be a substrate for PSA. Incubation with purified PSA caused hydrolysis of the 52 kDa SPMI precursor into smaller polypeptides (Fig. 4). After 3 hours of incubation, the 52 kDa precursor was transformed into smaller fragments of 8-31 kDa and the 52 kDa protein was no longer detectable. Only the 31, 19 and 16 kDa fragments were recognized by the SPMI antibody. Following 24 hours of incubation, the 31 kDa fragment was further degraded into smaller fragments and the staining intensity of the 16 and 17 kDa fragments had decreased with the concomitant appearance of 10-13 kDa fragments.

Microsequencing

Attempts to obtain the N-terminal sequence of the 52 kDa SPMI precursor by Edman degradation failed even though the quantity of protein used greatly exceeded standard detection limits (> 100 picomoles), suggesting that the N-terminus was blocked. To

circumvent this problem, four different polypeptides produced by PSA digestion of SPMI precursor were submitted to microsequencing by Edman degradation in order to obtain internal sequences. The 17, 16 and 12 kDa polypeptides yielded three distinct sequences (Table III). Search among known protein sequences in the GenBank and Swiss-Prot data banks using the Blast protocol (Altschul et al., 1990) revealed that the three sequences experimentally obtained matched perfectly (100% identity) various internal amino acid sequences of a protein known as semenogelin. These three sequences corresponded respectively to residues 137-143, 45-50, and 85-90 of semenogelin I protein precursor (Lilja et al., 1989). Semenogelin II protein precursor (Lilja and Lundwall, 1992) was also highly homologous but contained a few mismatches. Other protein sequences in the databank also showed some limited homology to SPMI fragments but only the semenogelin I precursor showed 100% identity for all three sequences obtained. N-terminal sequencing of the other SPMI polypeptide (19 kDa fragment) also failed to yield any sequence.

Amino acid analysis

The values obtained from amino acid composition analysis of the purified 52 kDa SPMI precursor are shown in table IV. Each value was compared with those calculated from the semenogelin I protein precursor cDNA sequence (Lilja et al., 1989), since the results obtained from N-terminal sequencing suggested homology with semenogelin. The amino acid composition of SPMI precursor and semenogelin appear nearly identical thus confirming the results of N-terminal sequence and the observed deviations are within reasonable experimental error. Most notable are the abundance of glutamine/glutamate, serine, and glycine residues, as well as the basic amino acids lysine and histidine. The protein contains relatively few

hydrophobic and aromatic residues. Moreover, it contains only a single cysteine residue and appears devoid of methionine.

Mass spectrometric analysis

Determination of the mass of purified SPMI precursor by electrospray-ionization mass spectrometry yielded a value of 49, 677 Da. When this value is corrected to take into account alkylation of a single cysteine residue with iodoacetamide prior to purification (-57 Da), the calculated mass of the unmodified SPMI polypeptide backbone becomes 49, 620 Da. This value is in good agreement with an expected mass 49, 607 kDa, calculated according to the cDNA sequence of semenogelin (Lilja et al., 1989).

Effect of SPMI precursor on intact human spermatozoa.

Treatment of washed and highly motile human spermatozoa with purified SPMI precursor caused a rapid dose-dependent decrease in sperm motility with a 50% relative motility inhibition (IC_{50}) observed at 240 U/ml (Fig. 5A). Complete inhibition of sperm motility was observed at 500 U/ml. Sperm velocity also progressively decreased with increasing SPMI concentration (Fig. 5B). Inactive seminal protein fractions obtained from the flow through of S-Sepharose chromatography (Fig. 1A, peak 2) had no effect on sperm motility, when used at the same concentration as the SPMI precursor, when compared to samples containing bovine serum albumin (not shown). The inhibitory effects of SPMI precursor were dependent on the time of contact with spermatozoa. Lower doses of SPMI achieved full inhibition when the incubation period was prolonged (data not shown). To assess reversibility, immobilized spermatozoa were washed by centrifugation on a mini Percoll gradient (Fig. 5C). Under

these circumstances, 60 ± 6% of the total number of spermatozoa layered on the Percoll gradient were recovered at the bottom of the gradient, and their motility was partially restored (80 ± 20% of control).

When SPMI precursor was pre-incubated with PSA for 24 hours, the precursor was transformed into lower mass polypeptides (as in fig. 4) which was accompanied by a 76 ± 4% decrease in SPMI precursor activity, as measured by the demembranated-reactivated sperm assay (Fig. 6A). On the other hand, when incubated with zinc chloride-inactivated PSA, all SPMI precursor activity was conserved. When intact spermatozoa were added to PSA-treated SPMI precursor, motility was only partially reduced (58% of control), while untreated SPMI precursor, or SPMI precursor incubated with zinc chloride-inactivated PSA completely inhibited sperm motility (Fig. 6B). PSA and zinc did not significantly affect sperm motility.

DISCUSSION

In humans, freshly collected seminal vesicle fluid is highly viscous and often variable in consistency. Proper solubilization of proteins in urea is therefore mandatory, in order to completely recover the 52 kDa SPMI precursor prior to chromatography. The purification procedure for SPMI precursor is relatively simple and straightforward. However, proper reduction of disulfide bonds and covalent modification of thiol groups prior to chromatography is essential to achieve successful purification of the 52 kDa SPMI form. In the absence of disulfide reduction, significant quantities of contaminants of intermediate molecular mass were carried over from the first to the second peak during the HPLC run (data not shown) suggesting that disulfide bridges exist between these SPMI molecules. Disulfide reduction and covalent modification of SPMI precursor, with iodoacetamide, did not result in any significant change in SPMI activity (data not shown). Immunodetection of the basic polypeptides eluting from the S-Sepharose column revealed that most polypeptides whose masses are lower than the 52 kDa polypeptide are antigenically related to SPMI and are likely degradation products of the 52 kDa precursor (Robert and Gagnon, 1994). The following chromatographic step on the reverse phase-HPLC column allowed the separation of the 52 kDa SPMI precursor from these smaller SPMI peptides.

The relatively small purification factor (2 to 5-fold) for the 52 kDa SPMI precursor and its predominance as reflected by the relative staining intensity of the 52 kDa protein band in seminal vesicle secretions and seminal coagulum proteins (Fig. 1C and 2C) indicates that it is the major component of human seminal vesicle fluid and semen coagulum. It may account for as much as 20 and 50% of the total proteins in these sources, respectively. The observation that the protein is active in denatured and reduced form suggests that a linear

segment of the molecule is responsible for the sperm motility inhibiting activity rather than a three-dimensional structure.

The observation that SPMI precursor was not recovered during gel filtration experiments performed in a physiological buffer indicates that macromolecular aggregates likely form under these conditions. The fact that denaturing elution conditions such as 8 M urea or the presence of detergent allowed normal elution of SPMI from the gel filtration column, suggests that SPMI molecules interact together via hydrophobic or other non-covalent interactions and form macromolecular aggregates of very high molecular weight under physiological conditions. A similar phenomenon was reported for bovine seminal plasma proteins that are also detrimental to sperm motility (Al-Somai et al., 1994). Elution of SPMI precursor at a position corresponding to a mass of 105 kDa suggests that the protein may retain a particular three-dimensional structure, in the presence of 8M urea, causing elution earlier than would normally be expected.

PSA hydrolyzed the SPMI precursor into multiple polypeptides with an associated loss of most of its activity as observed in whole semen after ejaculation (Robert and Gagnon, 1994). Moreover, most peptides released by PSA hydrolysis of purified SPMI precursor have masses corresponding to those observed in liquefied semen (Robert and Gagnon, 1994), strongly suggesting that PSA is the main enzyme responsible for SPMI degradation in semen, at least in the early post-ejaculatory stages. However, the possibility that other proteases also participate in the phenomenon cannot be ruled out (Robert and Gagnon, 1994). Interestingly, not all polypeptides resulting from hydrolysis of SPMI precursor were recognized by the SPMI antiserum, suggesting that the antigenic determinant may be limited to a specific portion of the precursor molecule. This phenomenon is to be expected since the SPMI

antiserum was originally raised against a 19 kDa SPMI fragment isolated from liquefied semen (Iwamoto and Gagnon, 1988a). This hypothesis also receives support from the fact that two of the immunoreactive polypeptides that were sequenced (16 and 12 kDa) appear to have a N-terminal located within the first 100 amino acid residues of semenogelin. On the other hand, the 19 kDa polypeptide, which is also immunoreactive but failed to yield a sequence by Edman degradation, likely corresponds to the blocked N-terminal peptide.

The basic character of SPMI precursor observed during purification is reflected in its amino acid composition which is rich in the basic residues histidine and lysine. The presence of only one cysteine residue in the SPMI precursor prevents the formation of intramolecular disulfides but as demonstrated previously for semenogelin, this residue appears to be actively involved in intermolecular disulfide bridge formation (Lilja and Laurell, 1985). The fact that the N-terminal amino acid sequences of three different SPMI precursor fragments are identical to those of internal segments of semenogelin, as well as the close agreement between the mass of SPMI precursor measured by mass spectrometry and that predicted for semenogelin from its cDNA sequence, strongly suggest that these two proteins are identical. Moreover, the amino acid composition of SPMI precursor is almost identical to that of semenogelin and the differences observed are within reasonable experimental error.

Semenogelin is a protein that was originally characterized as the main structural component of semen coagulum (Lilja et al., 1989). It was found to be present in semen coagulum as a 52 kDa molecule that gets degraded to lower mass forms, as semen liquefies (Lilja and Laurell, 1985). No significant homology to other known protein sequences was found. Two different expected masses of 49, 607 and 49, 621 Da were previously reported from the semenogelin cDNA sequence (Lilja et al., 1989). This difference of 14 Da corresponded to

a single nucleotide discrepancy observed between the sequence obtained from two different semenogelin clones, in which a serine residue at position 56 is substituted for a threonine. This variation may represent a DNA sequencing error or a true genetic variant (Lilja et al., 1989). However, the presence of a serine residue at this position, as reported from the sequence of a peptide isolated from seminal plasma that appears to be derived from residues 45-136 of semenogelin (Li et al., 1985) argues in favor of the predominance of the lower mass form. N-terminal pyroglutamination of semenogelin was previously suspected (Lilja et al., 1989), and this hypothesis is reinforced by the present failure to obtain sequence from the 52 kDa SPMI precursor and a 19 kDa SPMI fragment. In addition, N-terminal pyroglutamination was reported in a 26 amino acid peptide isolated from human seminal plasma that has an amino acid sequence identical to residues 1 to 26 of semenogelin (Kausler and Spiteller., 1992). Pyroglutamination of the N-terminal glutamine residue would thus reduce the expected mass of the precursor to 49, 590. The measured mass, for SPMI precursor, after correction for carboxymethylation of a single cysteine residue, is 49, 620. This 30 Da difference is within reasonable experimental error (< 0.1 % of total mass of polypeptide) and likely represents oxidation of the two tryptophan residues present in the precursor (+32 Da). This phenomenon is known to occur frequently during sample handling. The good agreement between the expected and measured mass of SPMI precursor suggests the absence of any extensive post-translational modification of the precursor besides N-terminal pyroglutamination.

All three peptides whose sequence was obtained from PSA-catalyzed SPMI precursor cleavage contain a N-terminal amino acid adjacent to the hydrophobic residues leucine and tyrosine preferentially cleaved by chymotrypsin-like proteases. Our results thus support the

previously reported chymotrypsin-like specificity of PSA, obtained by comparing amino acid residues homology in the active site of PSA with other members of the serine-family of proteases or from studies using synthetic substrate and various proteins (Watt et al., 1986; Akiyama et al., 1987; Schaller et al., 1987; Lundwall and Lilja, 1987; Christensson et al., 1990). More recently, computer modeling of the 3-D structure of PSA also demonstrated that its active site structure was most similar to that of chymotrypsin (Vihinen, 1994). However, the present results provide for the first time a direct demonstration of the chymotrypsin-like activity of PSA on a physiological substrate and confirm that the origin of several peptides previously isolated from seminal plasma that are believed to be derived from semenogelin are genuine products of PSA hydrolysis of the precursor protein (Lilja et al., 1984; Li et al., 1985; Lilja et al., 1989).

Purified SPMI precursor at a dose of 500 U/ml completely inhibited the motility of intact spermatozoa within 5 minutes and SPMI activity levels in semen immediately after ejaculation usually exceed that level (Robert and Gagnon, 1994). This result contrasts with previous findings using a degraded 19 kDa SPMI form isolated from liquefied seminal plasma which showed that 1600 U/ml were necessary to inhibit completely the motility of intact spermatozoa (Iwamoto and Gagnon, 1988b). This difference suggests that at an identical level of activity (as measured in the demembranated-reactivated sperm assay), SPMI precursor is 3 to 4-fold more potent than the lower mass hydrolysis products with respect to the inhibition of motility of intact spermatozoa. This phenomenon likely reflects a stronger affinity of SPMI precursor for its target site on spermatozoa as compared with the degraded forms. The effects of SPMI on sperm motility appear to be reversible since immobilized spermatozoa can resume movement following washing. This argues in favor of a cell surface

mediated effect whose exact nature remains to be investigated. Pre-incubation of SPMI precursor with PSA contributed to decrease the activity of SPMI precursor (demembranated-reactivated sperm assay) and to reduce its effects on sperm motility, demonstrating that SPMI precursor is the active component and that at equal amounts, the degraded forms have much less effect on sperm motility than the precursor form. Considering the abundance of the 52 kDa precursor in seminal vesicle fluid and seminal coagulum (10-17 mg/ml), the concentrations of SPMI precursor protein used to inhibit completely sperm motility ($\leq$ 5 mg/ml) are compatible with its concentration in seminal plasma immediately after ejaculation. Various cationic proteins have been shown to be detrimental to sperm motility (Iwamoto et al., 1992; Al-Somai et al., 1994; Shivaji, 1988). Cationic proteins are known to bind to sperm membranes and may act on spermatozoa in a manner similar to their action on bacterial membranes (Farley et al., 1989). However, the high isoelectric point (pI >8.0) of SPMI precursor does not appear to be the main characteristic that is responsible for its activity since the highly basic cytochrome c (pI of 10.5) at a concentration up to 10 mg/ml had no significant effect on sperm motility. Moreover, a peptide derived from a cationic antimicrobial protein was recently shown to have specific sperm immobilizing activity not solely due to the charge of the peptide (D'Cruz et al., 1995).

The results of the present study offer a new explanation for the reported low sperm motility in freshly ejaculated and coagulated semen (Tauber and Zaneveld, 1981). Immediately following ejaculation, SPMI activity levels in semen normally ranges between 500-2000 U/ml (Robert and Gagnon, 1994; 1995) and sperm motility is thus probably inhibited due to SPMI activity on spermatozoa, as observed in the present experiment. As the precursor gets degraded through PSA hydrolysis, SPMI activity is gradually reduced, providing a likely

explanation for the progressive increase in sperm motility during semen liquefaction. Physical restraint to sperm movement due to the entrapment of the cells in a web of protein aggregate in the coagulum may be partially responsible for the immobilization of spermatozoa, but such a phenomenon is unlikely to cause the complete arrest of flagellar movement as was observed in the present study using SPMI precursor. The purified precursor at concentrations up to 1000 U/ml (≈10 mg/ml), remains readily soluble, yet all flagellar beating is completely arrested suggesting a specific effect on spermatozoa rather than immobilization via a network of extracellular fibers. The physiological importance of the temporary sperm immobilization following ejaculation remains to be established. However, as demonstrated previously, the absence of proper processing of SPMI precursor by PSA appears related to fertility problems in men with non-liquefying semen (Robert and Gagnon, 1994).

In summary, we have developed a simple method for the purification of a natural sperm motility inhibitor present in human semen. This factor is the major protein of seminal vesicle secretions and seminal coagulum and appears to be an important regulator of sperm motility after ejaculation. All its characteristics indicate that it is identical to semenogelin, the predominant structural component of semen coagulum. The road is now paved for further investigating its molecular processing by PSA after ejaculation and analyze its function in the reproductive process.

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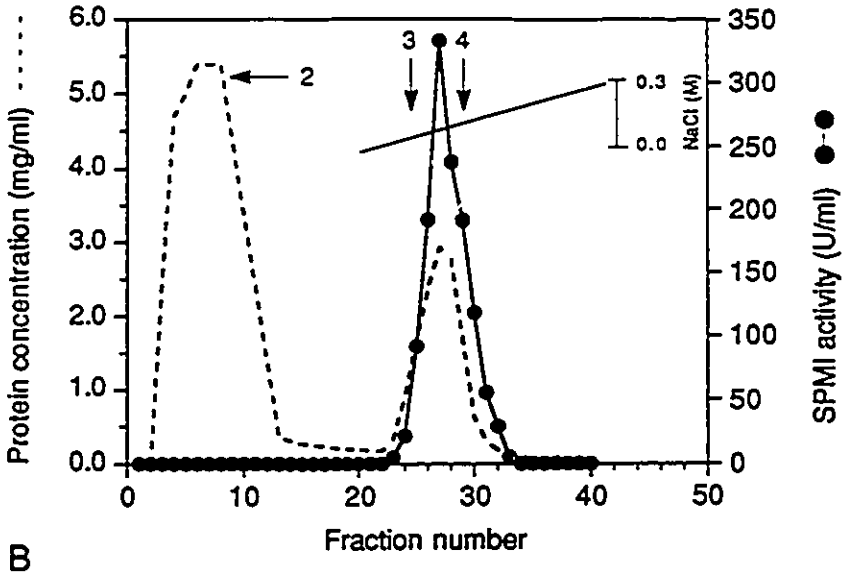
Figure 1. Purification of SPMI precursor from seminal vesicle fluid.

A) S-Sepharose chromatography. Solubilized seminal vesicle fluid was centrifuged at 10,000 x g for 15 minutes. The resulting supernatant was loaded on to a 5 X 100 mm S-Sepharose column and separated using a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Baie d'Urfé, QC, Canada) equilibrated in HSS containing 1 M urea (buffer A) at a flow rate of 1 ml/min. The column was washed in buffer A to remove any unbound material. Bound proteins were eluted by a linear gradient of 0 to 300 mM NaCl in buffer A at a flow rate of 1 ml/min. SPMI activity was assayed as described in Materials and Methods. Numbers identify different protein peaks whose content is shown in panel C. **2.** Flow through proteins, **3.** Early eluting components **4.** Late eluting components. This chromatogram is representative of a series of 8 similar procedures.

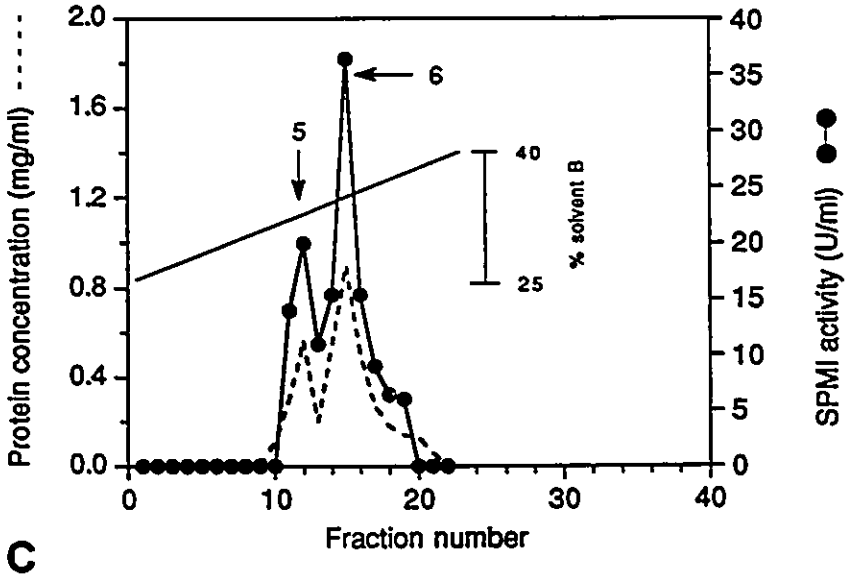
B) C_4 reverse-phase high performance liquid chromatography (RP-HPLC). Selected active fractions from the S-Sepharose chromatography (peak 4) were pooled, made up to 0.1% TFA and loaded on a Vydac (The Separations Group, Hesperia, CA) semi-preparative C_4 protein column (10 X 300 mm, 10μm beads, 300 Å pore size) equilibrated in solvent A (0.1% TFA). Proteins were eluted with a linear gradient from 25 to 40% of solvent B (80% acetonitrile/0.1% TFA) over 30 minutes at a flow rate of 3 ml/min. Eluted fractions were dried in a rotary evaporator and stored at -70°C until assayed. Numbers identify different protein peaks whose content is shown in panel C. This chromatogram is representative of a series of 8 similar procedures.

C) Electrophoretic analysis of chromatography fractions. Aliquots of fractions from the various chromatographic peaks of panel **A** and **B** were mixed with SDS sample buffer, heated to 95°C for 3 minutes and analyzed by SDS-PAGE and immunoblotting: **1.** Solubilized seminal vesicle fluid. Numbers **2 to 6** correspond to fractions isolated from the numbered peaks of panel **A** and **B**. No immunoreactive proteins were observed when the samples were probed with pre-immune serum.

A


Protein concentration (mg/ml)
SPMI activity (U/ml)
Fraction number
NaCl (M)
0.3
0.0
2
3
4

B


Protein concentration (mg/ml)
SPMI activity (U/ml)
Fraction number
% solvent B
40
25
5
6

C

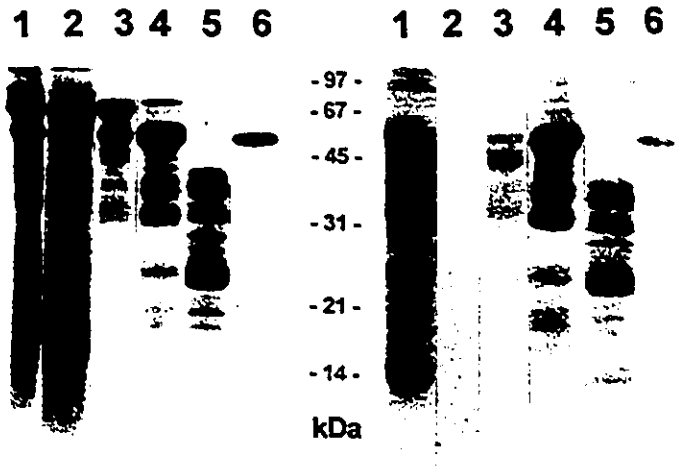
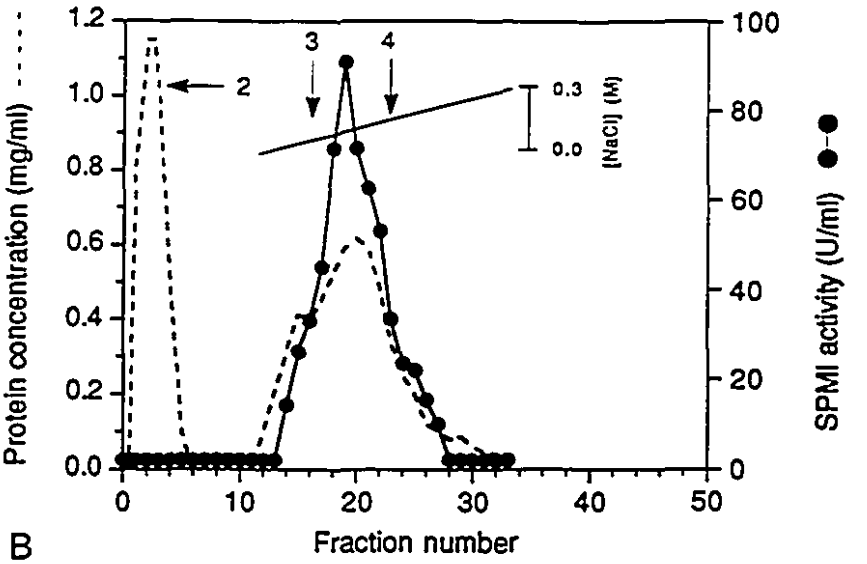

1 2 3 4 5 6
1 2 3 4 5 6
-97-
-67-
-45-
-31-
-21-
-14-
kDa
SDS-PAGE
Immunoblot

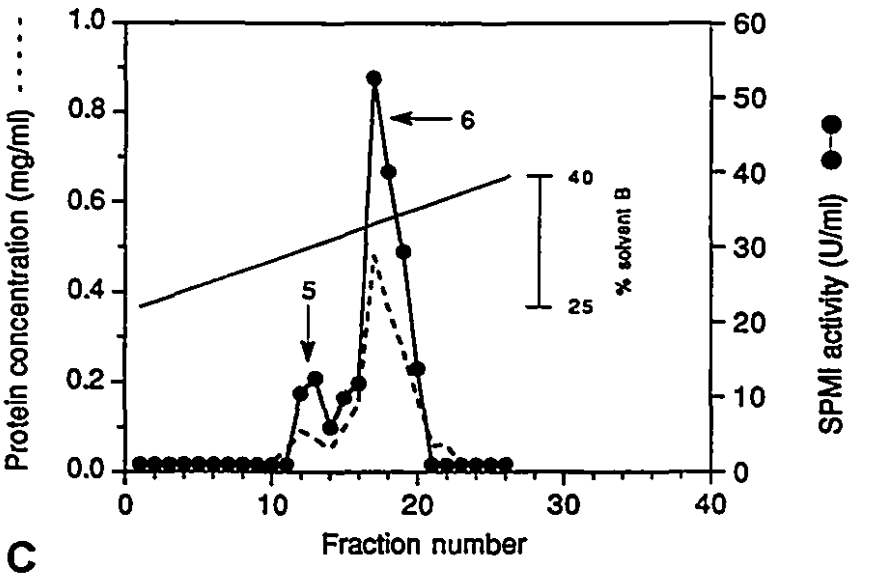
Figure 2. Purification of SPMI precursor using washed semen coagulum.

The procedure was the same as in Figure 1 except that solubilized seminal coagulum was used instead of seminal vesicle fluid (see the legend to Figure 1). **A) S-Sepharose chromatography B) C4 RP-HPLC C) Electrophoretic analysis of chromatography fractions.** The chromatograms are representative of a series of 5 similar procedures.

A


Protein concentration (mg/ml)
SPMI activity (U/ml)
Fraction number
[NaCl] (M)
0.3
0.0
2
3
4

B


Protein concentration (mg/ml)
SPMI activity (U/ml)
Fraction number
% solvent B
40
25
5
6

C

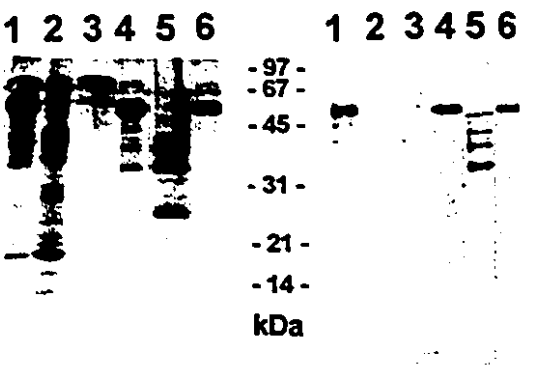

1 2 3 4 5 6
1 2 3 4 5 6
97
67
45
31
21
14
kDa
SDS-PAGE
Immunoblot

Figure 3. Gel filtration chromatography of the purified SPMI precursor.

The chromatography was performed on a Superdex-200 (Pharmacia Biotechnology, Baie d'Urfé, QC, Canada) column under native (HSS) and denaturing (8M urea/HSS) conditions. The column was eluted at a flow rate of 0.5 ml/min using a Pharmacia FPLC system. Protein elution was monitored at 280 nm. **1.** Void volume, **2.** Gamma-globulin, **3.** Bovine serum albumin, **4.** ovalbumin, 5. cytochrome c.

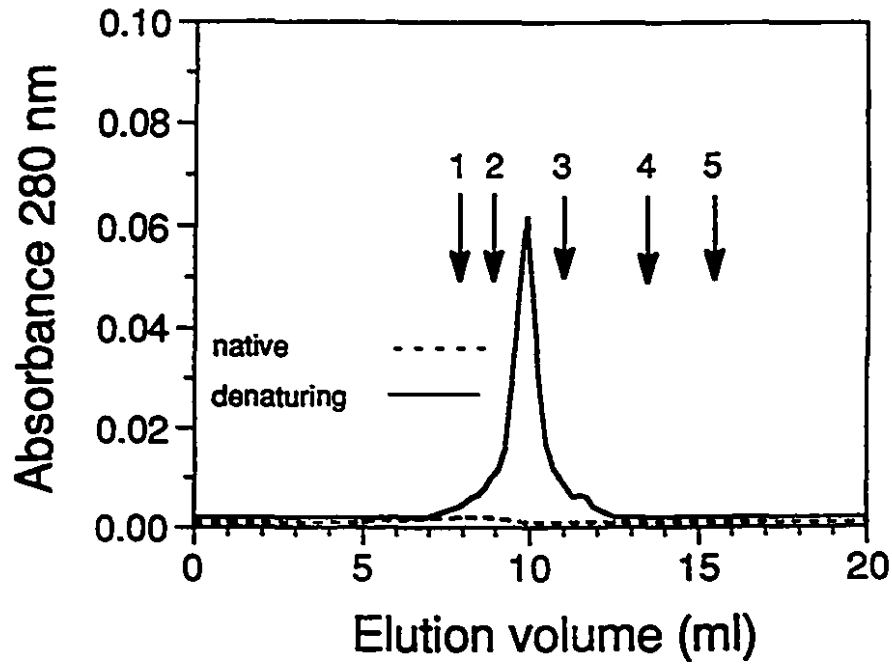

0.10
0.08
0.06
0.04
0.02
0.00
Absorbance 280 nm
1 2 3 4 5
native
denaturing
0
5
10
15
20
Elution volume (ml)

Figure 4. Digestion of the SPMI precursor by PSA.

The purified SPMI precursor was solubilized in HSS, pH 8.0 at a concentration of 0.5 mg/ml and incubated with PSA at room temperature at a 1:50 enzyme:substrate ratio (w/w). Following 3 or 24 hours of incubation the sample was solubilized in SDS sample buffer and analyzed by SDS-PAGE and immunoblotting. C. Control purified SPMI precursor without PSA. The 33 kDa band observed at 3 and 24 hours corresponds to PSA. The bands marked with a dash were selected for N-terminal sequencing.

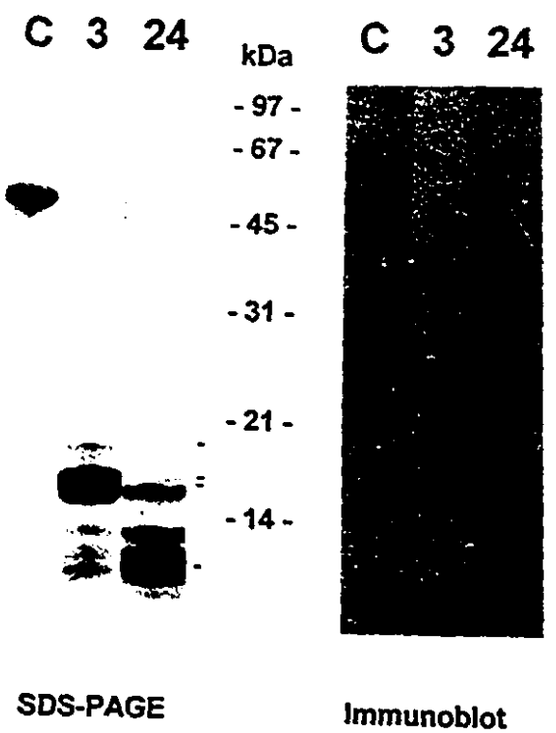
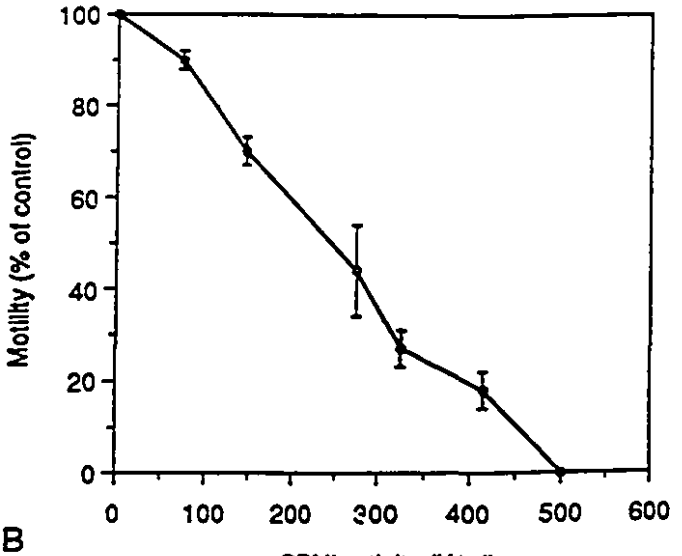

C 3 24
kDa
- 97 -
- 67 -
- 45 -
- 31 -
- 21 -
- 14 -
C 3 24
SDS-PAGE
Immunoblot

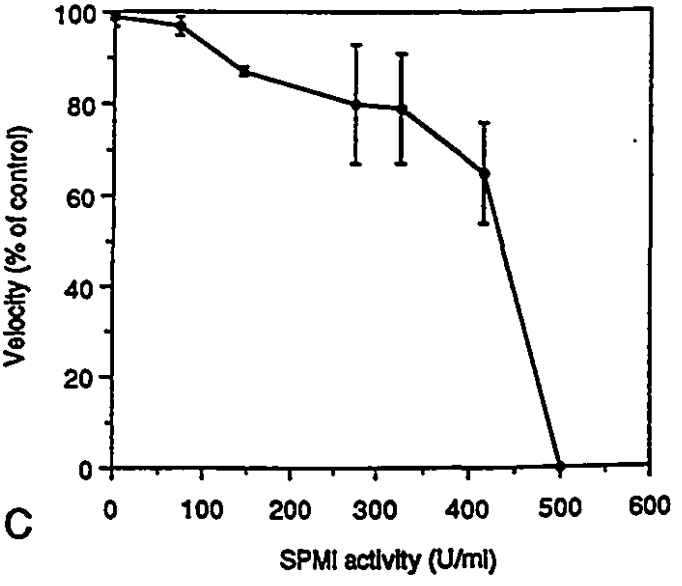
Figure 5. Effect of SPMI precursor on the motility of intact spermatozoa.

Spermatozoa were incubated for 5 minutes with various concentrations of purified SPMI precursor or control proteins (bovine serum albumin or cytochrome c (pI of 10.5), at the same protein concentration) in HBS buffer. The final concentration of spermatozoa in all samples was 50 X 10^6cells/ml. Motility was then assessed as described in Materials and Methods. Motility values are expressed as percentage of controls ± S.D. The percentage of motility in the control sample was 60 ± 10% (n=3). **A) Effect on sperm motility B) Effect on sperm velocity C) Effect of washing SPMI immobilized spermatozoa.** Following immobilization with 500 U/ml of SPMI precursor, spermatozoa were layered on a mini Percoll gradient (50µl of 65% Percoll and 20 µl of 95% Percoll in HBS) and centrifuged 10 minutes at 2000 X g. The bottom layer (20 ul) was recovered and motility assessed. Control samples containing BSA or cytochrome c at the same protein concentration underwent the same treatment. Values are expressed as relative motility ± S.D. compared to control samples (n=4).

A


Motility (% of control)
100
80
60
40
20
0
0
100
200
300
400
500
600
SPMI activity (U/ml)

B


Velocity (% of control)
100
80
60
40
20
0
0
100
200
300
400
500
600
SPMI activity (U/ml)

C

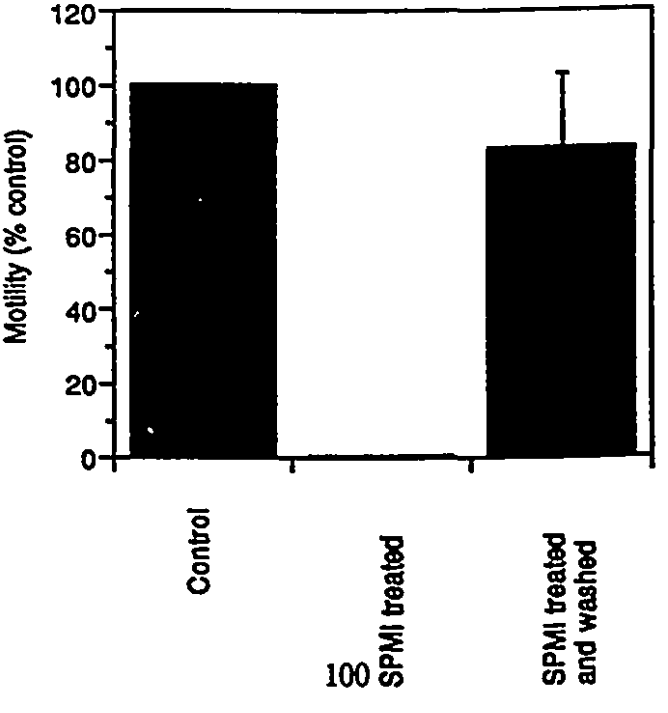
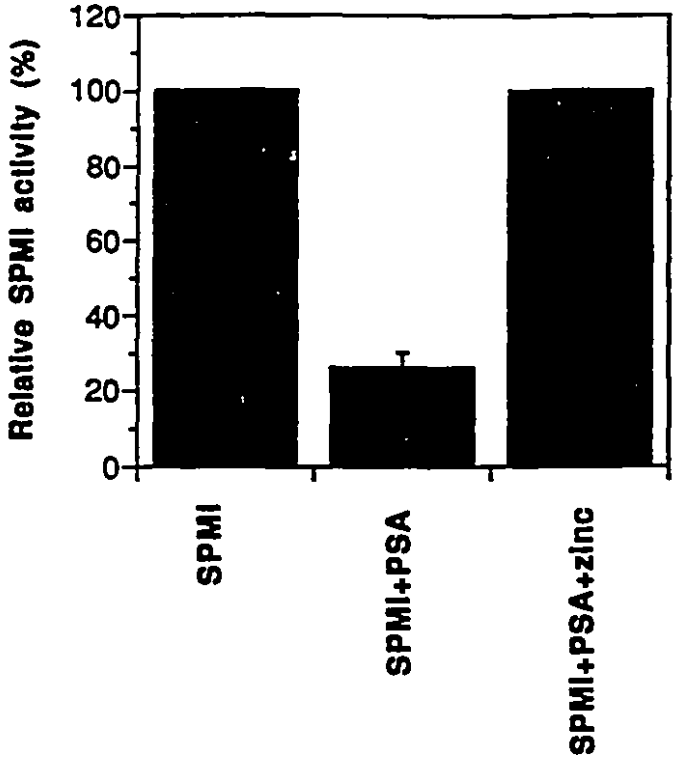

Motility (% control)
120
100
80
60
40
20
0
Control
100 SPMI treated
SPMI treated and washed

Figure 6. Effect of pre-incubating SPMI precursor with PSA. SPMI precursor was pre-incubated with PSA (20:1, w/w) or zinc-inactivated (10 mM zinc chloride) PSA. After 24 hours, SPMI activity was assayed as described in Materials and Methods. Results are expressed as relative SPMI activity ± S.D. compared to untreated SPMI precursor (n=4). **A) Effect on SPMI activity level. B) Effect on sperm motility.** SPMI precursor at 500 U/ml was pre-incubated with PSA (20:1, w/w) or zinc-inactivated (10 mM zinc chloride) PSA. After 24 hours, spermatozoa were added to the mixture and sperm motility was measured after 5 minutes as described in Materials and Methods. Results are expressed as relative motility ± S.D., compared to control samples containing BSA or cytochrome c at the same protein concentration as SPMI precursor (n=4).

A


Relative SPMI activity (%)
120
100
80
60
40
20
0
SPMI
SPMI+PSA
SPMI+PSA+zinc

B

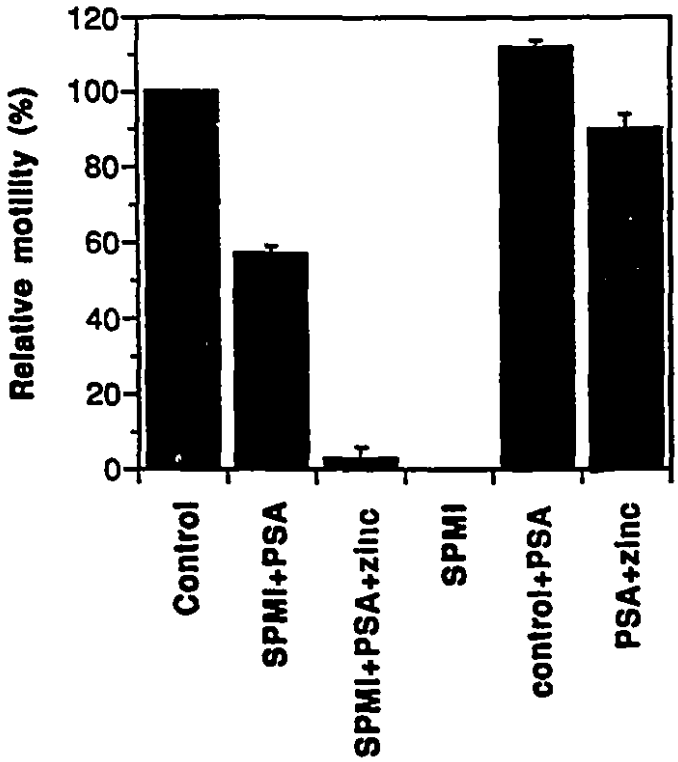

Relative motility (%)
120
100
80
60
40
20
0
Control
SPMI+PSA
SPMI+PSA+zinc
SPMI
control+PSA
PSA+zinc

Table I. Purification of SPMI precursor from seminal vesicle fluid.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Recovery rate (%)
Diluted seminal vesicle fluid	2736	187.0	14.6	1.0	100
S-Sepharose FF chromatography	2058	36.0	57.2	3.9	75
C4 reverse-phase chromatography	318	4.4	73.0	5.0	12

Table II. Purification of SPMI precursor from washed semen coagulum.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Recovery rate (%)
Solubilized coagulum	1110	24.0	46.3	1.0	100
S-Sepharose FF chromatography	540	6.1	88.0	1.9	49
C4 reverse-phase chromatography	203	2.0	100.0	2.2	18

Table III. N-terminal sequence analysis of SPMI.

Polypeptide	N-terminal sequence	Corresponding semenogelin sequence
18 kDa	SNTEERL	137 ...SQY**SNTEERL**W...
17 kDa	*TYHVDA	45 ...IQY**TYHVDA**ND...
12 kDa	*HNKQEG	85 ...QLL**HNKQEG**RD...

* Polypeptides that are immunoreactive with SPMI antiserum.

Table IV. Amino acid composition of SPMI precursor and semenogelin.

Amino acid	SPMI precursor*	Semenogelin†
Aspartic acid/asparagine	10.1	9.8
Threonine	4.4	4.3
Serine	11.4	12.3
Glutamic acid/glutamine	21.1	20.7
Proline	2.7	2.3
Glycine	9.0	8.9
Alanine	3.1	3.0
Cysteine	0.2	0.2
Valine	3.9	4.1
Methionine	0.1	0.0
Isoleucine	3.1	3.2
Leucine	5.8	5.5
Tyrosine	2.9	3.2
Phenylalanine	1.3	1.4
Histidine	7.0	7.1
Lysine	8.8	9.1
Arginine	4.6	4.6
Tryptophan	ND	0.5

* Numbers represent percent relative values of total amino acids. The values for SPMI precursor are averages of three different determinations. Tryptophan residues in the sequence were not determined due to destruction during acid hydrolysis.

† Semenogelin composition is according to its published cDNA sequence (Lilja et al., 1989).

Chapter V

Proteolytic processing of a sperm motility inhibitor precursor/semenogelin by prostate-specific antigen

Martin Robert and Claude Gagnon

Preface

In chapter IV, we provided evidence that SPMI precursor is identical to semenogelin, the main structural component of semen coagulum. The protein can inhibit the motility of intact spermatozoa in a reversible manner, supporting its role in the temporary sperm immobilization observed in semen after ejaculation. Moreover, the protein appears to be cleaved in a chymotrypsin-like manner by prostate-specific antigen to generate some SPMI polypeptides similar to those found in liquefied semen. PSA is likely the main SPMI precursor processing enzyme in semen. Its activity thus appears very important for proper SPMI precursor processing and release of motile spermatozoa. In the following chapter we have characterized for the first time the activity of PSA on its major physiological substrate, studied the details of PSA-mediated SPMI precursor processing, and mapped the different polypeptides released by PSA hydrolysis to specific domains of the precursor molecule.

Proteolytic processing of a sperm motility inhibitor precursor/semenogelin by prostate-specific antigen

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SUMMARY

Human seminal plasma contains a protein that can inhibit sperm motility. This factor, named seminal plasma sperm motility inhibitor (SPMI) originates from the seminal vesicles as a very active 52 kDa precursor form. Following ejaculation, SPMI precursor is rapidly degraded in semen by prostatic proteases to be transformed into less active, low molecular mass forms. SPMI precursor was found to be identical to semenogelin, the predominant protein of semen coagulum. The prostatic kallikrein-like enzyme prostate-specific antigen (PSA), a marker widely used clinically for monitoring prostatic malignancies, appears to be the main SPMI precursor processing enzyme after ejaculation. However, little is known about the activity of this enzyme on its physiological substrates. The present study was aimed at characterizing the proteolytic processing of the SPMI precursor/semenogelin by PSA. Purified SPMI precursor/semenogelin was incubated with PSA in the presence or absence of protease inhibitors. The general serine protease inhibitors 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and phenylmethylsulfonyl fluoride (PMSF), the trypsin inhibitor, p-nitrophenyl guanidobenzoate, heavy metal cations (Zn^{2+} and Hg^{2+}) and the heavy metal chelator 1,10-phenanthroline partially or totally inhibited the proteolytic activity of PSA towards SPMI precursor/semenogelin. Under identical conditions, other proteins, such as bovine serum albumin, ovalbumin and casein were very poor substrates for PSA. The inhibitory effect of 1,10-phenanthroline on PSA-mediated degradation of SPMI precursor/semenogelin appeared to be mediated by the chelation of substrate associated ions. SPMI hydrolysis products were separated by reverse-phase high performance liquid chromatography, assayed for SPMI activity, and analyzed for immunoreactivity using various antibodies. The various PSA cleavage sites were identified by mass spectrometric analysis of the hydrolysis products. The

region responsible for SPMI activity was localized to the N-terminal portion of the molecule between residues 85 and 136. This portion of the precursor molecule also corresponded to the segment recognized by the SPMI antiserum. On the other hand, a monoclonal antibody against a seminal vesicle-specific antigen (MHS-5) recognized fragments derived from the central part of the SPMI precursor/semenogelin (residues 198-223). PSA hydrolysis occurred almost exclusively at either leucine or tyrosine residues, demonstrating directly for the first time a restricted chymotrypsin-like activity on a physiological substrate. The results suggest that PSA is the main enzyme responsible for the processing of SPMI precursor/semenogelin in human semen and that this protease manifests a strong specificity with respect to hydrolyzable substrates and sites of hydrolysis.

INTRODUCTION

Seminal plasma is formed at ejaculation when secretions from the various male accessory sex glands are combined. It contains numerous proteins and peptides that are products of active synthesis of the accessory glands (Mann and Lutwak-Mann, 1981). However, the role of most of these substances in the reproductive system remains poorly understood. Some factors have been associated with immunosuppressive activities, protecting spermatozoa during transit through the female reproductive tract (Kelly, 1991), while others act as capacitating/decapacitating factors (Bedford, 1983; Han et al., 1990). Seminal proteins have also been found to bind to the sperm surface (Mann and Lutwak-Mann, 1981), act as receptors during binding to the zona pellucida (Topfer-Peterson, 1994), and may play a role in the modulation of the acrosome reaction and fertilization (Chandonnet et al., 1990; Coronel et al., 1992).

Following spermatogenesis and maturation during epididymal transit, spermatozoa are stored in the cauda epididymis where they are maintained in an immotile state (Eddy and O'Brien, 1994). Motility is induced at ejaculation when spermatozoa are mixed with seminal plasma components (Lindholmer, 1974). However, immediately after ejaculation, human spermatozoa remain immotile, trapped in the semen coagulum (Tauber and Zaneveld, 1981). As semen liquefies, forward motility is progressively initiated. Paradoxically, the seminal plasma of various species contains factors detrimental to sperm motility (Iwamoto et al., 1992; Jeng et al., 1993; Al-Somai et al., 1994). One such factor, the seminal plasma sperm motility inhibitor (SPMI), was isolated from human seminal plasma (Iwamoto and Gagnon, 1988a). This 19 kDa protein inhibits the motility of intact spermatozoa in a dose-dependent manner (Iwamoto and Gagnon, 1988b). SPMI originates exclusively from the seminal

vesicles (Luterman et al., 1991) as a very active 52 kDa precursor form (Robert and Gagnon, 1994). After ejaculation, the precursor is partially inactivated by transformation into less active, low molecular mass forms by prostatic proteases (Robert and Gagnon, 1994). Recent evidence suggests that absence of normal SPMI precursor processing may result in poor sperm motility (Robert and Gagnon, 1995).

SPMI precursor has been purified from seminal vesicle fluid and coagulated semen and found to be a potent inhibitor of sperm motility when tested at levels comparable to those encountered in semen immediately after ejaculation (Robert and Gagnon, Chapter IV). This inhibitory effect is reversible and appears to be sperm surface-mediated since motility can be recovered after washing the immobilized cells. Once processed, the levels of SPMI activity are reduced, allowing the initiation of progressive sperm motility. Biochemical analysis revealed that the SPMI precursor is identical to semenogelin, the main structural protein of semen coagulum (Lilja et al, 1989; Robert and Gagnon, Chapter IV). SPMI precursor/semenogelin is a basic 49.6 kDa non-glycosylated protein. It contains a total of 439 amino acids and the only apparent post-translational modification consists of the presence of a pyroglutamine residue at its N-terminus (Lilja et al., 1989; Robert and Gagnon, Chapter IV).

Human seminal plasma is rich in proteolytic enzymes (Mann and Lutwak-Mann, 1981). One of these proteases, the prostate-specific antigen (PSA) has been extensively studied, and is widely used clinically in monitoring the growth of prostatic malignancies (Oesterling, 1991). PSA was originally purified from seminal plasma as a prostate-specific protein (Wang et al., 1979). Characterization of its primary structure by amino acid sequencing and cDNA cloning revealed that PSA is a 33 kDa glycoprotein with a high degree of sequence homology to the

kallikrein-family of proteases (Watt et al., 1986; Lundwall and Lilja, 1987; Schaller et al., 1987). In contrast to other members of the kallikrein family, its primary structure suggests a chymotrypsin-like activity, a claim supported by experimental evidence using synthetic substrates and various proteins (Watt et al, 1986; Akiyama et al., 1987; Schaller et al., 1987; Christensson et al., 1990). Moreover, computer modeling of the three-dimensional structure of PSA showed that its substrate specificity pocket is closely related to that of chymotrypsin (Vihinen, 1994). However, no direct characterization of PSA activity has yet been performed on physiological substrates. PSA can cleave SPMI precursor/semenogelin, the predominant protein in human seminal vesicle fluid, in a manner reminiscent of its degradation in semen. Preliminary results also demonstrate that some of the hydrolysis sites are consistent with a restricted chymotrypsin-like specificity (Robert and Gagnon, Chapter IV).

In the present study we have investigated the proteolytic processing of SPMI precursor/semenogelin by PSA. The results reveal that when compared with other proteins, SPMI precursor/semenogelin is preferentially hydrolyzed by PSA, in a non-classical chymotrypsin-like manner and that the sperm motility inhibiting activity of SPMI precursor/semenogelin resides within the N-terminal domain of the molecule.

MATERIALS AND METHODS

Materials

Glycine, N-2-hydroxyethyl piperazine N-2'-ethanesulfonic acid (HEPES), cyclohexylaminopropane sulfonic acid (CAPS), sodium dodecylsulfate (SDS), dithiothreitol (DTT), benzamidine, p-nitrophenyl guanidobenzoate (NPGB) were purchased from ICN Biomedicals (Montréal, QC, Canada); acrylamide, β-mercaptoethanol (β-ME), Coomassie blue, nitroblue tetrazolium chloride (NBT) and bromo-chloro-indolphosphate (BCIP) from Bio-Rad (Mississauga, ON, Canada); iodoacetamide, aprotinin, 1,10-phenanthroline from Sigma Chemical Co. (St-Louis, MO, USA); adenosine triphosphate (ATP), and phenylmethylsulfonylfluoride (PMSF), 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), leupeptin, pepstatin, N-α-tosyl-phenylalanine-chloromethyl ketone (TPCK), and tosyl-lysine-chloromethyl ketone (TLCK) from Boehringer Mannheim (Laval, QC, Canada). Ultrapure urea was obtained from Bethesda Research Laboratories (Bethesda, MD, USA); HPLC-grade trifluoroacetic acid (TFA) and acetonitrile from J.T. Baker (Toronto, ON, Canada), Percoll from Pharmacia (Baie d'Urfé, QC, Canada), MHS-5 monoclonal antibody from Humagen Fertility Diagnostics (Charlottesville, VA, USA) and alkaline phosphatase-conjugated goat anti-rabbit and anti-mouse IgG from Jackson Immunoresearch Laboratories (West Grove, PA, USA). PSA purified from human seminal plasma was obtained commercially from Scripps Laboratories (San Diego, CA, USA). All other chemicals were at least of reagent grade.

Purification of SPMI precursor/semenogelin

The purification of SPMI precursor/semenogelin was performed as previously described (Robert and Gagnon, Chapter IV). Briefly, seminal vesicle fluid or washed seminal coagulum

were diluted in an equal volume of HEPES saline solution (HSS, 25 mM HEPES, 100 mM NaCl, pH 8.0) containing 8 M urea to achieve complete solubilization. The proteins were reduced by the addition of crystalline DTT to a final concentration of 25 mM and incubated at room temperature for 30 minutes. Reduced disulfides were then covalently blocked by the addition of crystalline iodoacetamide to a final concentration of 125 mM followed by incubation at room temperature for another 30 minutes. The proteins were precipitated in 94% ethanol at -70° C, resuspended in 8 M urea/HSS, and stored at the same temperature until use. Reduced and blocked proteins were loaded on a 5 X 100 mm, S-Sepharose Fast Flow (Pharmacia, Laval, QC, Canada) packed column using a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Laval, QC, Canada) equilibrated in HSS containing 1 M urea (buffer A) at a flow rate of 1 ml/min. The column was washed in buffer A to remove any unbound material. Bound proteins were eluted by a linear gradient of 0 to 300 mM NaCl in buffer A at a flow rate of 1 ml/min. Selected fractions from the S-Sepharose chromatography were pooled, made up to 0.1% TFA and loaded on a Vydac (The Separations Group, Hesperia, CA, USA) semi-preparative C_4 protein column (10 X 300 mm, 10μm beads, 300 Å pore size) equilibrated in solvent A (0.1% TFA). Proteins were eluted with a linear gradient from 25 to 40% of solvent B (80% acetonitrile/0.1% TFA) over 30 minutes at a flow rate of 3 ml/min. Fractions containing the purified SPMI precursor/semenogelin were dried in a rotary evaporator and stored at -70°C until assayed.

Hydrolysis of proteins with PSA

All hydrolysis reactions with PSA (presence or absence of inhibitors) were performed in HSS buffer (25 mM HEPES pH 7.6, 100 mM NaCl) at a 1:50 enzyme/substrate ratio for 24 hours

at 25°C. Purified SPMI precursor/semenogelin and other protein substrates were used at a final concentration of 1 mg/ml.

Isolation of motile spermatozoa.

Semen was provided by healthy volunteers by masturbation into sterile containers after 3 days of sexual abstinence. After liquefaction, the semen was layered on a discontinuous Percoll density gradient made of 2 ml each of 20, 40, 65% Percoll and 0.2 ml of 95% Percoll buffered in HBS (25 mM HEPES, 130 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$ and 14 mM fructose, pH 8.0). Following centrifugation at 1300 x g for 30 minutes, highly motile and morphologically normal spermatozoa from the 65-95% Percoll interface and the 95% Percoll layer were recovered and combined.

SPMI activity assay

Aliquots of the different eluted fractions were dried in a rotary evaporator and dissolved in 40 µl of demembranation-reactivation medium (0.1% Triton X-100, 0.2 M sucrose, 0.025 M potassium glutamate pH 8.0, 0.035 M Tris-HCl pH 8.0 and 1 mM DTT, 0.5 mM Mg.ATP. Percoll washed human spermatozoa (2 µl) was added to the medium and reactivation of sperm motility was evaluated under a phase-contrast microscope. Fractions in which reactivation of motility did not occur were considered to contain SPMI activity.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins from seminal plasma and other fluids were separated by SDS-PAGE according to Laemmli (1970) using the Bio-Rad, Mini Protean II electrophoresis system, after

solubilization in SDS-sample buffer (62.5 mM Tris-Cl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0125% bromophenol blue). The stacking and running gels contained 5 and 15% acrylamide, respectively. The electrophoresis was carried out at a constant current of 20 mA for one hour. The gels were then stained with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol/10% acetic acid and destained successively in 40% methanol/10% acetic acid and 10% methanol/10% acetic acid.

Immunoblotting and immunodetection

Following electrophoresis, proteins were electrotransferred from slab gels onto Nitroplus nitrocellulose membrane (Micron Separations Inc., Westboro, MA) for 40 minutes at 70 V in 10 mM CAPS and 10% methanol at pH 9.8. Following transfer, the membranes were air dried and stored at 4°C until use. Membranes were then first stained in 0.2% Ponceau S in 3% acetic acid to visualize proteins and molecular weight markers. After destaining in 10 mM Tris-HCl, 0.9% NaCl (TBS) pH 7.4, non-specific sites on the membranes were blocked with 10% swine serum in TBS containing 0.25% Tween-20 and 0.02% sodium azide (blocking solution) for 1 hour at room temperature. The membranes were then incubated for 1 hour with a rabbit anti-human SPMI antiserum generated against a 19 kDa SPMI form purified from human seminal plasma (Luterman et al., 1991) diluted 1:400 in blocking solution or the MHS-5 monoclonal antibody (1:2000) generated against a seminal vesicle-specific antigen (Herr et al., 1986). After four successive 10 minute washes in TBS containing 0.25% Tween-20, the membranes were incubated for 1 hour at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG or anti-mouse IgG diluted 1:2000 in blocking solution. Membranes were then washed four times in TBS containing 0.25%

Tween-20. Antigens were visualized by incubation in alkaline phosphatase buffer (100 mM Tris-HCl, 1 mM $MgCl_2$, pH 9.5) containing the substrates NBT and BCIP at 0.1 and 0.05 mg/ml, respectively. The reaction was terminated by washing the membrane in distilled water.

N-terminal amino acid sequencing

Hydrolysis products of SPMI precursor/semenogelin were separated by SDS-PAGE as described above and transferred onto Immobilon-P membrane (Millipore, Bedford, MA) for 40 minutes at 70 V in CAPS 10 mM pH 11.0 and 10% methanol. Individual polypeptide bands were excised from the membrane and inserted into the sample cartridge. The N-terminal amino acid sequence was determined by Edman degradation using a Porton Instruments automated gas-phase sequenator at the Protein Sequencing Facility of the Sheldon Biotechnology Center, McGill University, Montreal, QC.

Atmospheric pressure ionization mass spectrometry (API-MS)

Mass spectra were obtained in the positive mode on a triple stage mass spectrometer Model API-III (SCIEX, Toronto, ON, Canada). The samples were dissolved in 10% acetic acid and infused through a stainless steel capillary (100 µm I.D.) at a flow rate of 1µl/min. A stream of air (pneumatic nebulization) was introduced to assist in the formation of submicron droplets. These droplets were evaporated at the interface by nitrogen gas producing highly charged ions which were detected by the analyzer.

The system calibration was performed with the ammonium adduct ions of polypropylene glycol of known mass to charge ratios throughout the detection range of the instrument (0-2,470 atomic mass units). Instrument tuning, data acquisition and processing were controlled

by a computer system. Simple algorithms correlated the charges produced by the polypeptides to their molecular mass.

Identification of SPMI precursor/semenogelin peptides.

The complete semenogelin sequence (Lilja et al., 1989) was imported into the program Protein Analysis WorkSheet Version 5.1 (Robert Beavis, ©1995, New York University). The mass of cysteine 216 was adjusted to take into account alkylation with iodoacetamide before purification (+57 Da). Cleavage at leucine, tyrosine, phenylalanine and tryptophan residues was simulated to generate all possible combinations of hydrolysis products and their associated masses. The experimentally determined masses were then compared with the computer generated list of possibilities, allowing identification of the hydrolysis polypeptides along the precursor sequence.

Protein concentration determination

The concentration of proteins in all samples was measured with the bicinchoninic acid (Pierce Chemical Co., Rockford, IL, USA) assay following the procedure described by Smith et al. (1985) using bovine serum albumin as a standard.

RESULTS

Effect of protease inhibitors on processing of SPMI precursor/semenogelin by PSA

Incubation of SPMI precursor/semenogelin with purified PSA caused the hydrolysis of the 52 kDa precursor (Fig. 1) into multiple peptides with apparent masses below 20 kDa, while the purified 52 kDa SPMI precursor/semenogelin remained unchanged for 24 hours in the absence of enzyme. When PSA was pretreated with a series of protease inhibitors, various effects were observed (Fig. 1). The serine protease inhibitors PMSF and AEBSF, at a final concentration of 5mM, prevented the hydrolysis of most of the 52 kDa SPMI precursor/semenogelin. Only low levels of proteolytic products were visible when compared with that of the untreated SPMI precursor/semenogelin. The divalent heavy metal cations Zn^{2+} and Hg^{2+} at a concentration of 10 mM completely inhibited PSA activity, whereas Mn^{2+} and Ca^{2+} at the same concentration had no effect on PSA activity. The metal chelator 1,10-phenanthroline (50 mM) also prevented the degradation of SPMI precursor/semenogelin by PSA, while EDTA, at the same concentration, had no effect. The specific chymotrypsin inhibitor TPCK, like the specific trypsin inhibitors (TLCK, STI, leupeptin, benzamidine and aprotinin) had no effect on PSA activity. On the other hand, the trypsin titrant p-nitrophenyl guanidobenzoate inhibited PSA activity and caused a shift in molecular mass of SPMI precursor/semenogelin from 52 to 60 kDa. This shift in mass was accompanied by an apparent decrease in reactivity toward SPMI antiserum (Fig. 1). All inhibitors were proven effective when tested on their specific target proteases, indicating that they were all active (data not shown). The inhibitory effect of 1,10-phenanthroline was partially reversed when $ZnCl_2$ was added in increasing concentrations to samples containing 1,10-phenanthroline (Fig. 2).

Comparison of proteolytic activities of PSA and chymotrypsin on various substrates. Previous studies have reported that PSA displays chymotrypsin-like activity on synthetic substrates and proteins but that in comparison to chymotrypsin, this hydrolyzing activity is very low (Lilja, 1985; Watt et al., 1986; Akiyama et al., 1987; Schaller et al., 1987; Christensson et al., 1990). We have thus compared PSA activity with that of chymotrypsin on different proteins, including its predominant physiological substrate (SPMI precursor/semenogelin). Among the various protein substrates incubated with PSA, only SPMI precursor/semenogelin was significantly hydrolyzed into smaller fragments, while neither bovine serum albumin nor ovalbumin were visibly hydrolyzed by PSA (Fig. 3). Only very limited PSA hydrolysis of casein was observed. On the other hand, all substrates tested were hydrolyzed by chymotrypsin at a 1:50 enzyme/substrate ratio (w/w) yielding multiple bands of lower molecular masses. SPMI precursor/semenogelin was especially prone to hydrolysis by this enzyme. The proteolytic activity of chymotrypsin was also tested in the presence and absence of the heavy metal chelator 1,10-phenanthroline. The presence of 1,10-phenanthroline (50 mM) in the hydrolysis mixture greatly reduced chymotrypsin proteolysis when SPMI precursor/semenogelin was used as the substrate, but had no significant effect when other substrates were used. In contrast to its inhibitory effect on PSA activity, $ZnCl_2$ did not inhibit chymotrypsin activity toward any substrate tested (data not shown). However, as observed for PSA (Fig. 2), addition of $ZnCl_2$ (10 mM) partially reversed the inhibitory effect of 1,10-phenanthroline on SPMI precursor/semenogelin proteolysis by chymotrypsin.

Separation and analysis of SPMI precursor/semenogelin polypeptides released by PSA. To analyze the sites preferentially hydrolyzed by PSA, SPMI precursor/semenogelin was digested with PSA (1 : 50 enzyme:substrate ratio, w/w) for 24 hours (Fig. 4). The hydrolysis mixture was then injected on a C_4 reverse-phase HPLC column. Linear gradient elution with acetonitrile generated a series of peaks as depicted in Figure 5A. PSA was shown not to interfere with the elution of SPMI polypeptides since in a control run, it eluted at a concentration greater than 50% of solvent B (data not shown). The eluted fractions contained 1 to 3 peptides with apparent masses by SDS-PAGE between 6 and 20 kDa (Fig. 5B). An aliquot of each fraction was tested for SPMI biological activity using an *in vitro* assay on demembranated-reactivated spermatozoa, as described in Materials and Methods. Only the contents of peaks 1, 3, 4, and 5 were found to possess SPMI biological activity. To identify regions of SPMI precursor/semenogelin containing specific antigenic determinants, the contents of all fractions were also analyzed by immunoblotting using an SPMI antiserum generated against a 19 kDa SPMI fragment isolated from seminal plasma (Iwamoto and Gagnon, 1988a) and with the MHS-5 monoclonal antibody, generated against the seminal vesicle specific antigen (Herr et al., 1986). The reactivity of these antibodies toward SPMI polypeptides differed. The SPMI antiserum reacted with the series of consecutive early eluting fractions (1, 3 to 5) which also corresponded to the fractions containing the SPMI activity (Fig. 5A and C). The predominant immunoreactive peptides had apparent masses of 16, 15, 12, and 8 kDa. The contents of all other fractions did not demonstrate any reactivity with SPMI antiserum. On the other hand, the MHS-5 monoclonal antibody recognized four predominant polypeptides with apparent masses of 19, 10, 8, and 7 kDa (Fig. 5D) that eluted at acetonitrile concentrations higher than those recognized by the SPMI antiserum. Faint

immunoreactivity was also observed on a polypeptide with an apparent masses of 17 kDa (lane 13).

The content of all fractions demonstrating the presence of polypeptides, as judged by SDS-PAGE were subjected to ion-spray ionization mass spectrometric analysis. The polypeptides in these fractions produced distinct ionization patterns that allowed calculation of their respective masses with high accuracy (error < 0.1% of total mass). Since SPMI precursor was previously found to be identical to semenogelin (Robert and Gagnon, Chapter IV) and that PSA demonstrates restricted chymotrypsin-like activity, the experimentally determined masses were compared with the mass of peptides generated from a computer simulated digestion at the C-terminal of leucine, tyrosine, phenylalanine, and tryptophan residues of SPMI precursor/semenogelin according to the reported semenogelin sequence (Lilja et al., 1989) as described in Materials and Methods. Partial N-terminal sequence of some polypeptides was also obtained. Using this approach, the polypeptides in each fraction with masses matching those of the simulated cleavages could be assigned to a specific segment of the precursor molecule, thus identifying PSA cleavage sites. The results of the polypeptide mass spectrometric analysis are summarized in Table I. The measured masses of polypeptides were in excellent agreement with values calculated from the cDNA sequence of semenogelin. The mass variation was within 5 mass units per 10 kDa for the majority of polypeptides. Four different polypeptides had masses that were 14 to 20 Da higher than the expected masses. These higher masses are likely associated with the oxidation (+16 Da) of tryptophan residues 145 and 375, the only two oxidizable residues in the precursor, as observed previously (see chapter IV). In general, the measured mass for most peptides was significantly less than the apparent mass deduced by SDS-PAGE (Fig. 5, Table I).

The peptides identified in Table I cover the whole length of the precursor molecule, and show considerable overlap. The complete sequence of SPMI precursor/semenogelin identifying the observed hydrolysis sites is shown in Figure 6. Hydrolysis of SPMI precursor/semenogelin by PSA was incomplete since not all leucine, tyrosine and phenylalanine residues present in the precursor molecule were hydrolyzed. Certain residues in the vicinity of the cleavage sites appear to be conserved. In one instance, one or more serine residues found 3 to 6 residues upstream of the cleavage sites occur more than ten times and in another, a histidine or a glutamine residue immediately precedes cleaved tyrosine residues at eight different sites. Glutamine or glycine residues are also found 3 to 7 residues upstream of many cleavage sites. In addition, three different sites where adjacent leucine residues occur were hydrolyzed. An almost perfect repeat of 38 amino acids (only one mismatch) occurs at residues 259-296 and 319-356, and four different hydrolysis sites were observed at homologous positions in each of these repeats.

The information obtained from the mass and electrophoretic analyses of the polypeptides is represented schematically in Figure 7. The map reveals the part of the precursor that appears to contain the SPMI activity and the domains recognized by the various antibodies used. The polypeptides having SPMI activity correspond to those recognized by the SPMI antibody, and appear to define an area between residues 85 and 136 thus containing both the active region and the SPMI epitope. On the other hand, the polypeptides recognized by the MHS-5 antibody originate from a different, and more centrally located, portion of the precursor. Together these define an area between residues 198 and 223 that seem to constitute the epitope for this antibody. Regions corresponding to polypeptides previously isolated from human seminal plasma are also shown.

DISCUSSION

PSA has been shown to degrade readily SPMI precursor into multiple fragments of low molecular masses (Robert and Gagnon, Chapter IV). This degradation considerably reduces the inhibitory effect of SPMI on sperm motility. The primary structure of PSA suggests a chymotrypsin-like specificity (Schaller et al., 1987; Lundwall and Lilja, 1987). However, previous studies on the characterization of PSA activity using synthetic or non-physiological protein substrates suggested that the specificity of PSA is unusual in spite of its close similarity to chymotrypsin (Akiyama et al., 1987; Schaller et al., 1987; Christensson et al., 1990). For this reason, we decided to study the effect of various substances on PSA proteolytic activity measured on its main physiological substrate. The effect of the various protease inhibitors on PSA activity toward SPMI precursor/semenogelin suggests that while the active site of PSA is closely related to that of chymotrypsin (Vihinen, 1994), it displays major differences. The specific chymotrypsin inhibitor TPCK did not inhibit PSA activity. On the other hand, while most classical trypsin inhibitors had no effect on PSA activity, the trypsin titrant NPGB completely inhibited PSA activity. Beside binding to the active site of PSA, the trypsin inhibitor NPGB may have also covalently modified SPMI during the incubation since a shift in mass of the precursor and a loss of immunoreactivity with SPMI antiserum was observed after SDS-PAGE. NPGB is known to bind to serine residues in the active site of trypsin, and a similar binding to SPMI serine residues may have contributed to the modifications observed. Further studies will be required to confirm this hypothesis.

The specific inhibition of PSA activity by zinc and mercury suggests the possibility of a specific regulatory mechanism of PSA activity by heavy metals. It also highlights another difference with chymotrypsin which is not affected by heavy metals. Zinc is likely to be the

most physiologically relevant metal since its concentration averages 2 mM in seminal plasma (Mann and Lutwak-Mann, 1981). Most of the results on the effect of different protease inhibitors on PSA activity obtained in the present study are generally in agreement with those of previous studies on PSA activity. These studies, performed on non-physiological or synthetic substrates, showed inhibition of PSA activity by the active site reagents diisopropyl fluorophosphate (DFP) and PMSF and by zinc and mercury (Watt et al., 1986; Akiyama et al., 1987; Schaller et al., 1987). We also found in the present study that copper and nickel have an inhibitory effect on PSA activity similar to that of zinc and mercury (data not shown). The effect of the metal chelator 1,10-phenanthroline has not been reported previously, and was more puzzling. Since high concentrations of heavy metals can inhibit PSA activity, the inhibitory effect of the chelator was unexpected. A high concentration of the metal chelator was necessary to inhibit the degradation of SPMI precursor/semenogelin by PSA. The chelator could act by removing an ion associated with PSA and required for its activity. However, the effect of the metal chelator on PSA proteolysis of the other substrates tested was not evaluated since PSA did not readily hydrolyze them. We thus tested the effect of the chelator on chymotrypsin activity. The fact that 1,10-phenanthroline also affected the activity of chymotrypsin, but only when SPMI was used as the substrate suggests that the metal ion chelated by 1,10-phenanthroline is associated with the substrate rather than the enzyme. The possibility of a nonspecific effect of the metal chelator such as denaturation of the enzyme or substrate was ruled out since addition of 10 mM zinc to samples containing 1,10-phenanthroline partially restored both PSA and chymotrypsin activities toward SPMI precursor/semenogelin. Taken together these observations suggest that SPMI may be a zinc binding protein and that removal of SPMI-bound metal by chelators would reduce the

susceptibility of SPMI to proteolysis, possibly by inducing structural changes. SPMI precursor/semenogelin is rich in histidine, a known zinc binding amino acid (Robert and Gagnon, Chapter IV and Figure 6). This hypothesis is reinforced by the results of previous experiments demonstrating that the major zinc ligand in seminal plasma is the predominant coagulum protein having a molecular mass identical to SPMI/semenogelin (Frenette et al., 1989). The concentration of free zinc in seminal plasma is thus likely to be important for proper processing of SPMI precursor/semenogelin after ejaculation. High concentrations of free zinc are likely to inhibit PSA activity and thus degradation of the precursor, while a lack of zinc may protect SPMI from proteolysis. The latter effect is likely related to the arrest of semen liquefaction and SPMI/semenogelin degradation observed when 1,10-phenanthroline is added to freshly ejaculated semen (Lilja and Laurell, 1985; Robert and Gagnon, 1995). PSA appears to be highly specific with respect to hydrolyzable substrates as other proteins were poorly hydrolyzed. This may reflect a requirement for the presence of specific structures or consensus amino acid sequences in the substrates that are specifically recognized by PSA for hydrolysis. This hypothesis is reinforced by the fact that hydrolysis by PSA does not occur at all leucine and tyrosine residues in the precursor sequence and that certain amino acids in the vicinity of many PSA cleavage sites appear conserved. The activity of PSA was much lower than that of chymotrypsin under the conditions used. These results agree with those of previous studies showing that PSA activity was 200 to 4000 times lower than that of chymotrypsin (Watt et al., 1986; Akiyama et al., 1987). While the lower activity of PSA on most proteins may be an intrinsic property of the enzyme, it is also possible that a proportion of the PSA used in these experiments, which is purified from human seminal plasma, may be partially complexed with endogenous protease inhibitors (Christensson et al.,

1990; Laurell et al., 1992) or partially inactivated during its purification.

The analysis of SPMI polypeptides by mass spectrometry demonstrated that PSA exhibits a restricted chymotrypsin-like specificity. The sites of PSA hydrolysis were almost exclusively limited to leucine and tyrosine residues. The mass of only one polypeptide was consistent with hydrolysis at phenylalanine. Moreover, none of the peptides obtained were consistent with cleavage at tryptophan residues. The hydrolysis preference of PSA thus contrasts with that of chymotrypsin which preferentially hydrolyzes tyrosine, phenylalanine and tryptophan, and only to a lesser extent leucine residues. It also differs from the specificity of the other members of the kallikrein family of proteases which hydrolyze proteins at basic residues in a trypsin-like manner (Christensson et al., 1990). Previous studies have also associated trypsin-like activity to PSA (Lilja, 1985; Watt et al., 1986). However, this activity was later shown to originate from the presence of a contaminant enzyme in the PSA preparation (Akiyama et al., 1987; Christensson et al., 1990). The preference of PSA for hydrolysis at hydrophobic residues is explained by the presence of a serine residue in the bottom of the substrate specificity pocket, as in chymotrypsin (serine-183), whereas trypsin-like enzymes have an aspartic acid residue at the homologous position (Lundwall and Lilja, 1987; Schaller et al., 1987). In the latter, this aspartic acid residue favors electrostatic interactions with the positively charged arginine and lysine residues preferentially hydrolyzed by kallikrein proteases whereas the small side chain of the serine residue in PSA leaves space for bulky hydrophobic residues (Vihinen, 1994). The present results suggest that additional properties of the PSA active pocket may further increase the specificity of PSA when compared with chymotrypsin. It is also possible that, through tertiary structure alterations of the substrate, the presence of substrate bound-zinc near hydrolysis sites increases susceptibility of the

substrate to hydrolysis by PSA. In that context it is interesting to note that four different cleavage sites occurring at tyrosine residues are adjacent to a histidine residue.

Analysis of the different polypeptides with respect to SPMI activity and reactivity with the various antibodies provided information about different domains of the precursor. The fact that only polypeptides derived from a common region located between amino acid 85 and 136 contained SPMI activity suggests that the active site is located within this specific segment of the precursor molecule. An identical conclusion can be drawn for the epitope recognized by the SPMI antiserum. The presence of the epitope in that same region reflects the fact that the SPMI antiserum was originally raised against a 19 kDa active SPMI form isolated from liquefied seminal plasma (Iwamoto and Gagnon, 1988a). The two short peptides representing residues 1 to 25 and 26 to 44 of SPMI precursor/semenogelin could not be detected by SDS-PAGE or immunoblots. These are likely too small to stain well by Coomassie blue and are likely lost during polyacrylamide gel fixation and/or electroblotting. Thus, the possibility that this region (residues 1 and 44) might also be recognized by the SPMI antiserum cannot be ruled out. On the other hand, the polypeptides recognized by the MHS-5 monoclonal antibody appear to define a segment of the SPMI precursor/semenogelin located between residues 198 and 223. This monoclonal antibody was originally produced against washed human spermatozoa in an attempt to produce sperm-specific antibodies but it was later found to recognize a sperm-coating antigen secreted by the human seminal vesicles and thus termed seminal vesicle-specific antigen (Herr et al., 1986). The characteristics and fate of the seminal vesicle-specific antigen (SVSA) during semen liquefaction suggest that it is identical to SPMI/semenogelin (McGee and Herr, 1987; Lilja, 1989).

It is noteworthy that some of the fragments released by PSA hydrolysis of SPMI precursor/semenogelin correspond to polypeptides that had previously been purified from seminal plasma. The 5754 Da (residues 85-136) peptide containing the SPMI active site corresponds to the seminal basic polypeptide of 52 residues purified by Lilja and co-workers (1984) and to α-inhibin-52, an inhibin-like peptide previously isolated from seminal plasma (Li et al., 1985). The 10, 356 Da polypeptide (residues 45-136) corresponds to another inhibin-like peptide, α-inhibin-92 (Li et al., 1985). In addition, another seminal plasma peptide containing a N-terminal pyroglutamine residue, was previously characterized (Kausler and Spiteller, 1992), and corresponds to the 2770 Da peptide (residues 1-25) identified in the present experiments. These findings thus directly demonstrate that these polypeptides are derived from PSA hydrolysis of SPMI precursor/semenogelin. The possibility that SPMI precursor/semenogelin undergoes alternative processing by other seminal proteases cannot be ruled out. However, the present results support the notion that PSA is the main SPMI precursor/semenogelin processing enzyme in the early stage after ejaculation (Szecsi and Lilja, 1993; Robert and Gagnon, Chapter IV).

In summary, the present results provide for the first time a direct characterization of PSA activity on its major physiological substrate. PSA displays a non-classical and restricted chymotrypsin-like activity and appears specific with respect to substrate. The zinc concentration in seminal plasma is likely important in regulating SPMI precursor/semenogelin processing by PSA. The sperm immobilizing activity of the precursor molecule appears to reside within the N-terminal region of the precursor molecule. The hydrolysis of SPMI precursor/semenogelin by PSA releases multiple polypeptides that may have different functions in the reproductive process.

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Figure 1. Effect of protease inhibitors on PSA hydrolysis of SPMI precursor/semenogelin.

Various protease inhibitors were pre-incubated with PSA for 18 hours. SPMI precursor/semenogelin was then added and hydrolysis was performed for 24 hours. The reactions were stopped by the addition of SDS-sample buffer and heating to 95°C for 5 minutes and the proteins were analyzed by SDS-PAGE and immunoblotting as described in Materials and Methods. **A)** Coomassie blue stained gel. **B)** Immunoblot probed with an SPMI antiserum generated against a 19 kDa SPMI fragment. The concentrations of inhibitors, metals and chelators used were: AEBSF and PMSF, 5 mM; $ZnCl_2$, $HgCl_2$, $CaCl_2$, and $MnCl_2$, 10 mM; benzamidine, 1,10-phenanthroline and EDTA, 50 mM; NPGB 10 mM; leupeptin, 15 μM; TPCK and TLCK, 1 mM; STI, 1 mg/ml and aprotinin 0.1 mg/ml.

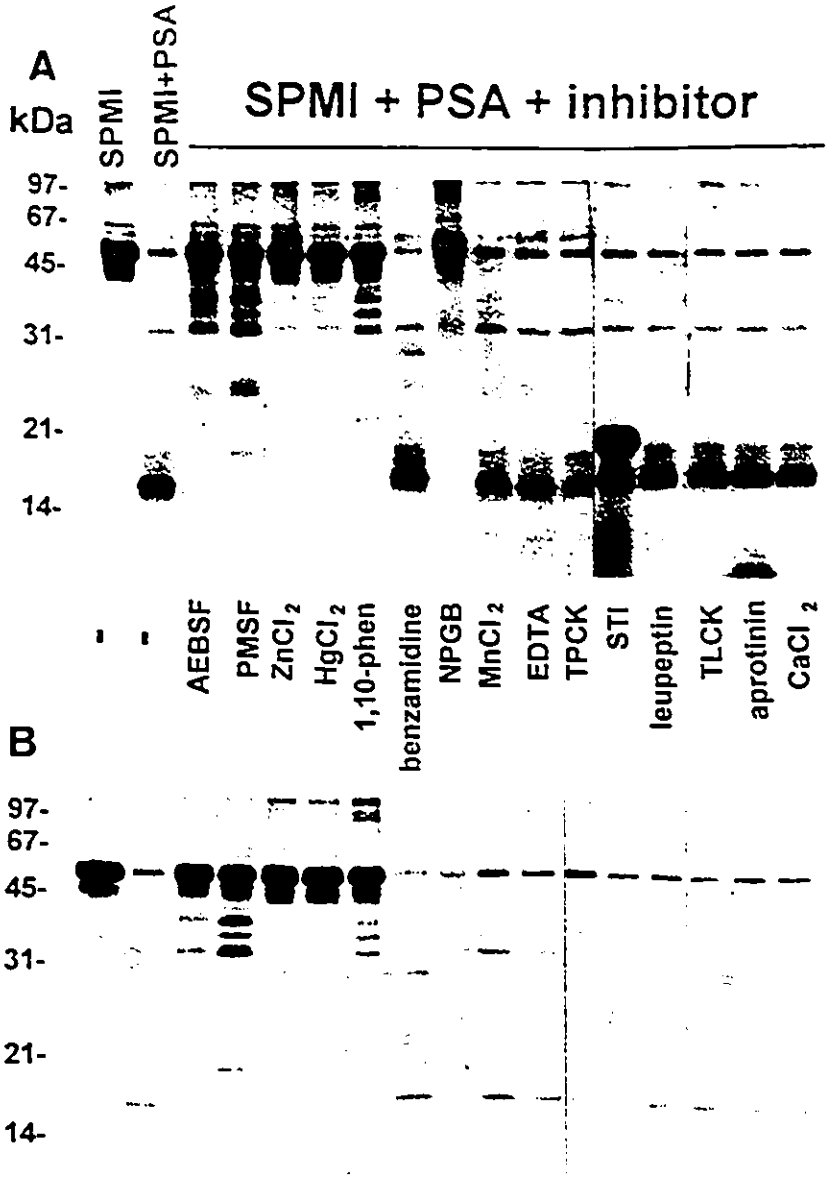

A
kDa
SPMI
SPMI+PSA
SPMI + PSA + inhibitor
97-
67-
45-
31-
21-
14-
-
-
AEBSF
PMSF
ZnCl2
HgCl2
1,10-phen
benzamidine
NPGB
MnCl2
EDTA
TPCK
STI
leupeptin
TLCK
aprotinin
CaCl2
B
97-
67-
45-
31-
21-
14-

Figure 2. Reversal of 1,10-phenanthroline effect on SPMI precursor/semenogelin degradation by PSA.

SPMI precursor/semenogelin was incubated with PSA in the presence of 1,10-phenantroline (50mM). After 10 minutes $ZnCl_2$ was added and the mixture was incubated for 24 hours. The reactions were stopped by the addition of SDS-sample buffer and heating to 95°C for 5 minutes. Samples were analyzed by SDS-PAGE as described in Materials and Methods. **1)** Control SPMI precursor/semenogelin. **2)** SPMI precursor/semenogelin and PSA in absence of 1,10-phenanthroline. **3)** SPMI precursor/semenogelin and PSA with 50 mM 1,10-phenanthroline. SPMI precursor/semenogelin and PSA with 50 mM 1,10-phenanthroline + **4)** $ZnCl_2$ 0 .1 mM **5)** $ZnCl_2$ 0.3 mM **6)** $ZnCl_2$ 3 mM **7)** $ZnCl_2$ 10 mM.

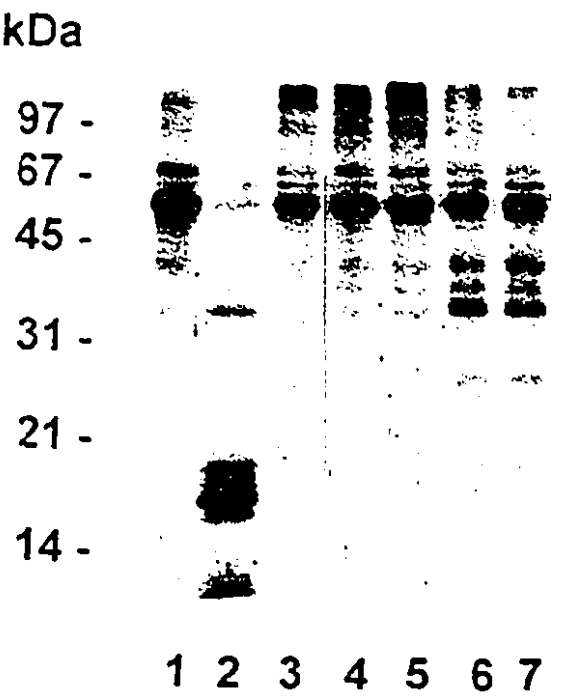

kDa
97 -
67 -
45 -
31 -
21 -
14 -
1 2 3 4 5 6 7

Figure 3. Activity of PSA and chymotrypsin on various substrates.

Various protein substrates were incubated with PSA (1:50 enzyme:substrate, w/w) or chymotrypsin (1:100 enzyme:substrate, w/w), in the presence or absence of 1,10-phenanthroline (50 mM), for 24 hours. The reaction was stopped by addition of SDS-sample buffer and the samples were analyzed by SDS-PAGE as described in Materials and Methods. **1)** Protein substrate without enzyme. **2)** Protein substrate + PSA. **3)** protein substrate + chymotrypsin **4)** protein substrate + chymotrypsin + 1,10-phenanthroline **5)** protein substrate + chymotrypsin + 1, 10-phenanthroline + $ZnCl_2$ 10 mM. Chymotrypsin (**C**), PSA (**P**).

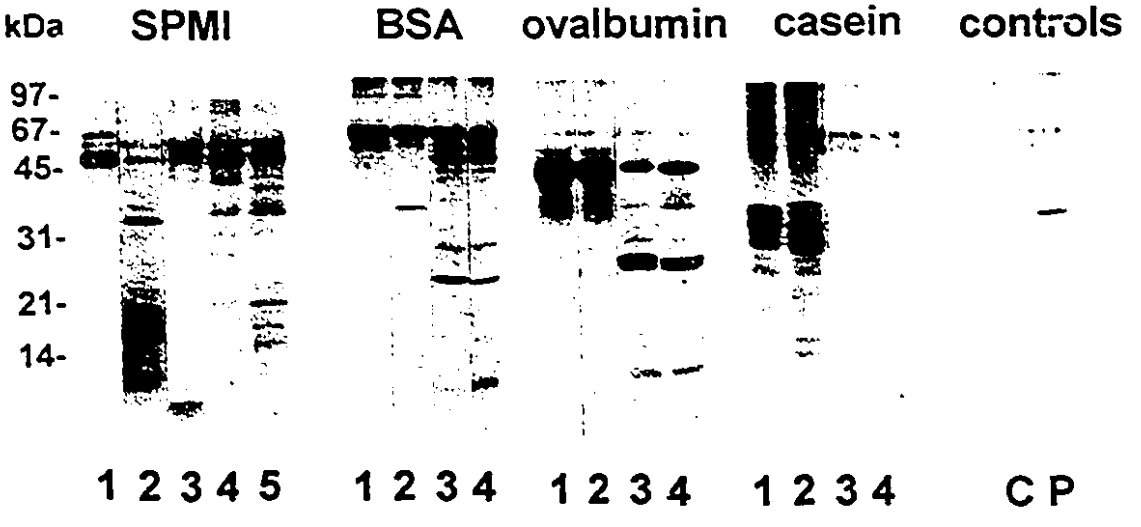

kDa
SPMI
BSA
ovalbumin
casein
controls
97-
67-
45-
31-
21-
14-
1 2 3 4 5
1 2 3 4
1 2 3 4
1 2 3 4
C P

Figure 4. Digestion of the SPMI precursor/semenogelin with PSA.

The purified SPMI precursor/semenogelin solubilized in HSS (800 μg) was incubated with PSA for 24 hours. An aliquot was taken, mixed with SDS-sample buffer and analyzed by SDS-PAGE as described in Materials and Methods. **1)** Control SPMI precursor/semenogelin without PSA. **2)** SPMI precursor/semenogelin with PSA

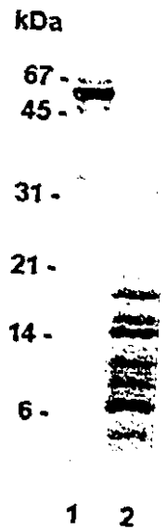
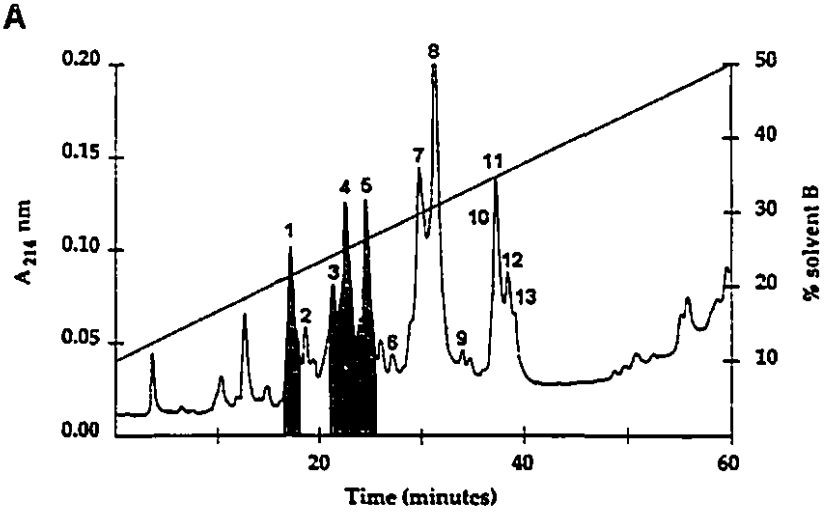

kDa
67 -
45 -
31 -
21 -
14 -
6 -
1
2

Figure 5. Separation and analysis of polypeptides released by hydrolysis of SPMI precursor/semenogelin by PSA.

A) The hydrolysis mixture shown in Fig. 4 was made up to 0.1% TFA and injected on C_4 reverse phase column (10 X 300 mm, 10μm beads, 300 Å pore size) equilibrated in solvent A (0.1% TFA). Peptides were eluted with a linear gradient of 10 to 50% solvent B (0.1% TFA in 80% acetonitrile) over 60 minutes. An aliquot of each fraction was dried and analyzed for SPMI biological activity using the demembranated-reactivated sperm assay. Fractions that contain activity are highlighted.

Individual fractions from the HPLC separation of hydrolysis fragments were dried in a rotary evaporator and analyzed by SDS-PAGE using the tricine based buffer system according to Schägger and von Jagow (1987) using 3% and 16.5% acrylamide for the stacking and resolving gels respectively. Proteins were stained in Coomassie Blue or electrotransferred to a nitrocellulose membrane as described in Materials and Methods. The numbers correspond the numbered peaks of panel A. Numbers 10 and 11 correspond respectively to the early and late eluting portion of the same peak at 37 minutes. Number 13 corresponds to the shoulder peak at 39 minutes. B) Coomassie blue stained gel. C) Immunoblot probed with SPMI antiserum (1:400) raised against a 19 kDa SPMI fragment isolated from seminal plasma D) Immunoblot probed with MHS-5 monoclonal antibody (1:2000) generated against a seminal vesicle-specific antigen (Herr et al., 1986).


A
0.20
0.15
0.10
0.05
0.00
A_{214} nm
50
40
30
20
10
% solvent B
20
40
60
Time (minutes)
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2
3
4
5
6
7
8
9
10
11
12
13

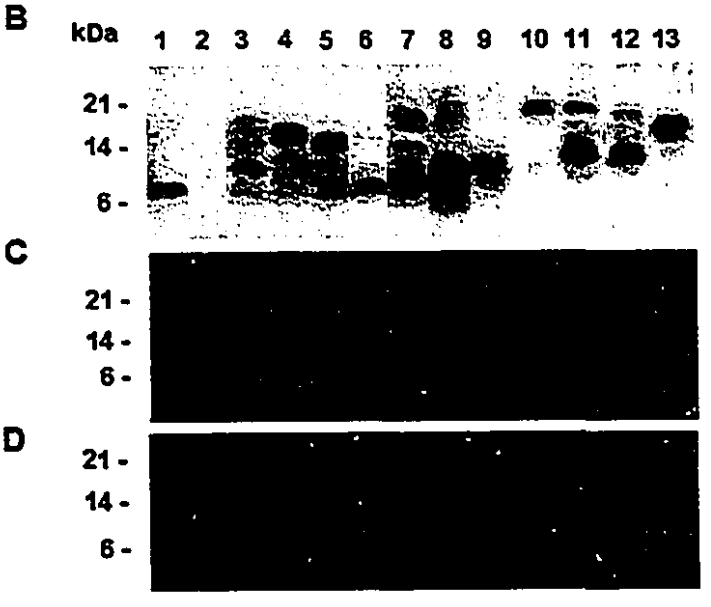
B
kDa
1 2 3 4 5 6 7 8 9 10 11 12 13
21 -
14 -
6 -
C
21 -
14 -
6 -
D
21 -
14 -
6 -

Figure 6. Complete sequence of SPMI precursor/semenogelin.

The complete sequence of semenogelin (Lilja et al., 1989) is presented in the standard IUPAC, one-letter code for amino acid residues. The observed PSA cleavage sites are identified by an arrow. Residues that occur at an identical position relative to the cleavage sites in at least two different PSA hydrolysis sites are underlined. Histidine residues near the cleavage sites are doubly underlined.

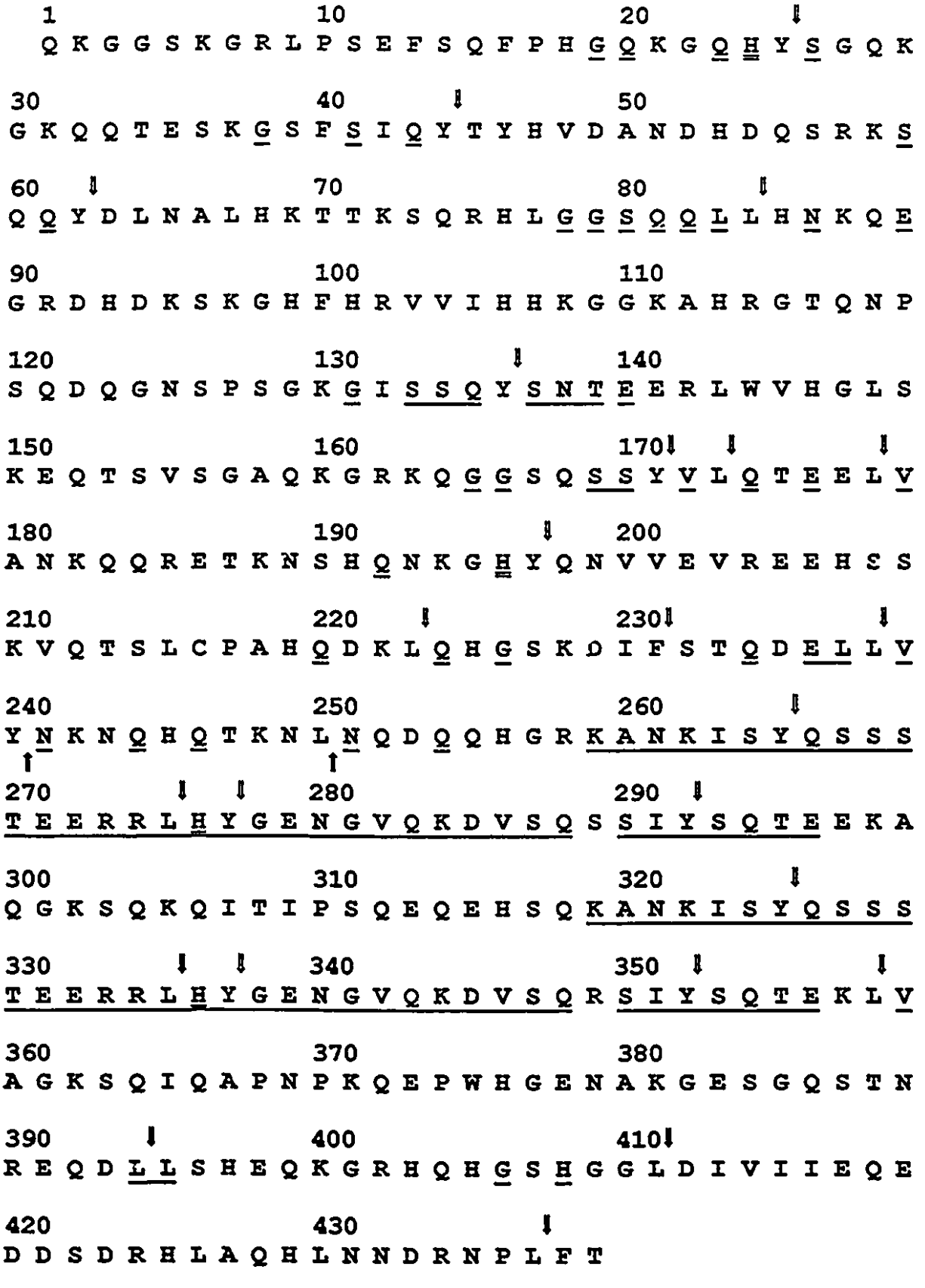

Figure 7. Schematic representation of SPMI precursor/semenogelin

The full SPMI precursor/semenogelin (residues 1 to 439) is shown with a carboxymethylated cysteine residue 216 (CM-cys) and a pyroglutamine residues at the N-terminus (pQ). The polypeptides identified in the present study are shown below the precursor with their N and C-terminus residue numbered. The order of presentation corresponds to that of Table I. The polypeptides shown in black contain SPMI activity and correspond to those recognized by the SPMI antiserum. The polypeptides recognized by the MHS-5 antibody are shown in gray. Polypeptides previously isolated from seminal plasma and characterized are shown above the precursor: α-inhibin-92 (Li et al., 1985), seminal basic protein or α-inhibin-52 (Lilja et al., 1984; Li et al., 1985) and a seminal peptide isolated by Kausler and Spiteller (1992).

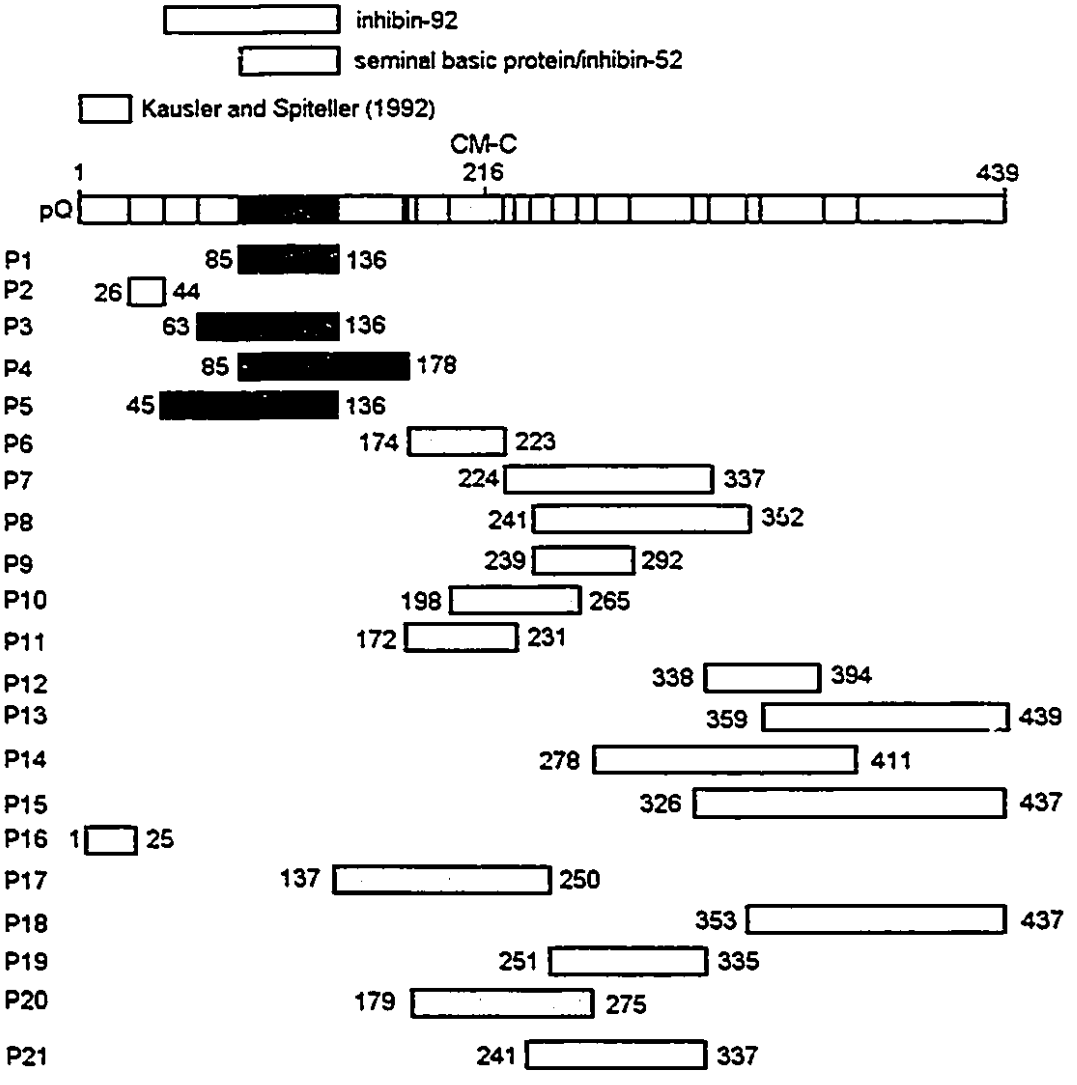
inhibin-92
seminal basic protein/inhibin-52
Kausler and Spiteller (1992)
CM-C
1
216
439
pQ
P1 85 136
P2 26 44
P3 63 136
P4 85 178
P5 45 136
P6 174 223
P7 224 337
P8 241 352
P9 239 292
P10 198 265
P11 172 231
P12 338 394
P13 359 439
P14 278 411
P15 326 437
P16 1 25
P17 137 250
P18 353 437
P19 251 335
P20 179 275
P21 241 337

Table I. Identification of PSA cleavage sites and resulting SPMI peptides.

Peptide	Fraction number	Measured mass	Expected mass from cDNA[a]	Polypeptide sequence	N-terminal sequence	Immuno-reactivity
P1	1	5754	5753	85-136	HNKQE	SPMI
P2	2	2089[b]	2088	26-44		
P3	3	8185	8181	63-136		SPMI
P4	4	10370[c]	10356	85-178		SPMI
P5	5	10356	10355	45-136	TYHVD	SPMI
P6	6	5873	5869	174-223		MHS-5
P7	7	13234	13239	224-337		
P8	7	12933	12939	241-352		
P9	7	6319	6322	239-292		
P10	8	7958	7957	198-265	QNVVE	MHS-5
P11	8	7000	6995	172-231		MHS-5
P12	8	6272	6266	338-394		
P13	9	9083[c]	9065	359-439		
P14	10	14939[c]	14922	278-411		
P15	10	12640	12639	326-437		
P16	10	2770[b,d]	2786	1-25		
P17	11	13060[c]	13040	137-250	SNTEERL	MHS-5
P18	11	9505	9503	353-437		
P19	12	9773	9770	251-335		
P20	13	11381	11380	179-275		MHS-5[e]
P21	13	11279	11277	241-337		

[a] Mass modified to take into account alkylation of one cysteine residue (see Materials and Methods).
[b] Peptide not visible by SDS-PAGE.
[c] Peptide showing a mass difference corresponding to oxidation (+16 Da).
[d] Peptide showing a mass difference corresponding to N-terminal pyroglutamination (-17 Da).
[e] Faint reactivity

Chapter VI

General discussion

Preface

The following discussion summarizes the main findings of the present study. The results of the different sections are integrated in an attempt to draw general conclusions on SPMI precursor, its molecular processing and physiological function.

General discussion

The results presented in this study clearly demonstrate that the presence of a 52 kDa SPMI precursor molecule in seminal vesicle fluid and its molecular processing in semen explain the rapid decrease in SPMI activity after ejaculation (Chapter II). The precursor was first transformed into intermediate molecular mass fragments (25-40 kDa) and subsequently into smaller fragments of 15-20 kDa. This transformation into smaller forms was accompanied by a large reduction in SPMI activity to levels that are compatible with normal sperm motility (150-200 U/ml). SPMI processing was reconstituted *in vitro* by incubating secretions isolated from the seminal vesicles and prostate gland, demonstrating the prostatic origin of the processing factor. The results showed that a serine protease inhibited by PMSF and a trypsin-like protease inhibited by benzamidine are responsible for SPMI processing in semen. However, the extensive SPMI precursor degradation taking place in the presence of benzamidine demonstrated that the protease inhibited by this compound was only responsible for limited proteolysis when compared with that of the PMSF-inhibited protease. In addition, the inhibitory effect of the metal chelator 1,10-phenanthroline on the degradation of SPMI precursor suggested that the same proteases may be metallo-dependent.

The similarities between the properties of SPMI processing after ejaculation and the extensive proteolysis occurring during semen liquefaction suggested a possible association between these phenomena. Moreover, abnormal SPMI processing after ejaculation could be responsible for the poor sperm motility associated with some cases of infertility. These possibilities were confirmed by analyzing SPMI in coagulated semen. All the SPMI activity was detected in the coagulated fraction of semen (Chapter III). In addition, SPMI precursor

and SPMI antigens of intermediate mass (20-38 kDa) were detected in this fraction. The possibility of a detrimental effect on sperm motility of abnormal SPMI processing after ejaculation was supported by observations made on semen samples obtained from a patient consulting for infertility. In these samples, which failed to liquefy spontaneously, very low sperm motility was associated with very high levels of SPMI activity (1500 U/ml), and the presence of nondegraded SPMI precursor forms. Addition of prostatic secretions to the nonliquefied semen samples resulted in a marked increase in sperm motility with a concomitant degradation of high mass SPMI forms and a decrease in SPMI activity. These findings offer an explanation for the known low sperm motility in coagulated semen shortly after ejaculation, and the progressive increase in sperm motility as SPMI precursor gets inactivated during semen liquefaction. While multiple anomalies may induce male infertility, these results suggest that improper SPMI processing may be responsible for infertility in a subset of asthenozoospermic patients whose semen fails to liquefy. The results also demonstrate that coagulated semen displays properties that are similar to seminal vesicle fluid with respect to SPMI activity and antigens, and could serve as a source of SPMI precursor.

6.1 Purification and properties of SPMI precursor

In order to gain further insight on SPMI characteristics and its molecular processing, a method to purify the SPMI precursor from the complex mixture of proteins present in seminal vesicle fluid and from washed seminal coagulum proteins was developed. One of the difficulties encountered during the purification of the SPMI precursor was in the removal of by-products of SPMI degradation present in the starting material. These by-products had an inherent tendency to co-elute with the 52 kDa precursor. Their successful removal was

achieved using high performance reverse-phase liquid chromatography. The strong staining intensity of the 52 kDa SPMI precursor polypeptide observed in SDS-PAGE of samples of seminal vesicle fluid and washed semen coagulum, together with the low enrichment factor (2- to 5-fold) for the SPMI precursor after purification, suggest that this protein may represent more than 20% and up to 50% of the total proteins in these sources of SPMI, respectively.

The purified precursor was incubated with PSA to investigate the possibility that PSA may be involved in SPMI processing in semen, and to obtain internal SPMI fragments from which N-terminal amino acid sequence could be derived. This particular enzyme was chosen because its characteristics corresponded well with those of the serine protease inhibited by PMSF that was found to be involved in SPMI precursor processing (Chapter II). It is also the most abundant protease in seminal plasma and appears to be the main enzyme responsible for semen liquefaction (Lilja, 1985; Lilja et al., 1987; McGee and Herr, 1988). Using purified preparations of SPMI precursor and PSA, we demonstrated that PSA performs proteolytic processing of SPMI precursor, similarly to what is observed in semen after ejaculation. Our results provided evidence that PSA cleaves SPMI precursor at residues that are consistent with a restricted chymotrypsin-like activity for PSA. The degradation of SPMI precursor resulted in a loss of most of its activity, as it occurs in whole semen after ejaculation.

Biochemical analysis of SPMI precursor and of some of its peptides provided evidence that SPMI is identical to semenogelin, the protein characterized as the main structural component of human semen coagulum. Although only partial amino acid sequence information was obtained for SPMI precursor, the characteristics of the processing of SPMI, its amino acid composition and its molecular mass make the likelihood of the proteins being identical

extremely high.

In physiological solution the purified SPMI precursor appeared to form aggregates of large molecular mass. This finding was expected, considering the proposed identity between SPMI precursor and semenogelin, and might be related with non-covalent interactions between proteins that appear to induce semen coagulation (as discussed below). The demonstration of the immobilizing effect of purified SPMI precursor on spermatozoa, at doses corresponding to those measured in coagulated semen early after ejaculation, strongly suggests that it is the factor responsible for the low motility of spermatozoa in coagulated semen. Its effects on sperm motility were reversible and appeared to be mediated at the sperm surface.

Experiments on the properties of SPMI precursor, its mass and processing by PSA were performed on both SPMI precursor purified from seminal vesicle fluid and seminal coagulum. Identical results were obtained for these two sources of SPMI precursor, suggesting that at ejaculation, the SPMI precursor does not undergo modifications by other components of semen, besides the hydrolysis by PSA.

In the last part of the present study, further information on the processing of SPMI precursor/semenogelin by PSA was obtained. Only the N-terminal domain of SPMI precursor/semenogelin appeared to contain the elements responsible for SPMI activity and reactivity with the antibody raised against a 19 kDa SPMI form present in liquefied seminal plasma. On the other hand, the epitope recognized by the MHS-5 monoclonal antibody appeared to originate from the central part of the precursor molecule. PSA cleaved SPMI precursor/semenogelin with a restricted chymotrypsin-like specificity, in contrast to the trypsin-like specificity of the other members of the kallikrein family of proteases, to which

PSA belongs. The unusual specificity of PSA is explained by the presence of a serine residue at the bottom of the substrate pocket where an aspartic acid residue is found in trypsin-like enzymes (Lundwall and Lilja, 1987). PSA also differs from chymotrypsin in its preference for leucine and tyrosine residues, while chymotrypsin predominantly cleaves at the aromatic residues phenylalanine, tryptophan, and tyrosine and, to a lower extent, at leucine residues. In addition, the classical chymotrypsin inhibitor TPCK did not affect PSA, while the trypsin inhibitor NPGB blocked PSA activity. Finally, zinc and other heavy metal ions inhibited PSA activity, while they did not affect chymotrypsin activity. PSA appeared more specific than chymotrypsin and did not readily hydrolyze most proteins tested. It is thus possible that PSA cleaves preferentially at peptide bonds surrounded by specific conserved residues as observed in SPMI precursor/semenogelin. These structures would need to be recognized by the enzyme in order for hydrolysis to occur and could explain the lower activity of PSA when compared with chymotrypsin. Together, these differences demonstrate that PSA is a unique processing enzyme that displays distinct properties in spite of its close relationship to other known serine proteases of the kallikrein family.

6.2 Possible mode of action of SPMI on sperm motility.

The exact mechanism by which SPMI exerts its action on spermatozoa is still unknown, and its elucidation remained beyond the scope of the present investigation. Nonetheless, it is possible to draw some inferences as to the nature of this activity. The 19 kDa form of SPMI purified from seminal plasma can inhibit the ATPase activity of the dynein of human demembranated-reactivated spermatozoa and that of dynein purified from bull spermatozoa (Iwamoto and Gagnon, 1988a). However, the inhibitory effect of SPMI on intact

spermatozoa (Iwamoto and Gagnon, 1988b and Chapter IV) is not well understood. Whether SPMI affects sperm motility by disturbing membrane structure, acting through a receptor on the cell surface or penetrating spermatozoa to interact directly with the axonemal dynein remains to be established. The possibility of a large protein readily penetrating into spermatozoa appears unlikely. However, an interesting study demonstrated the intracellular integration of a bull seminal vesicle protein into the mid-piece of spermatozoa and its binding to an acceptor protein on mitochondria (Aumüller et al., 1988). Moreover, the isolation and identification, in sperm nuclei, of a 19 kDa polypeptide that appears to originate from the N-terminal domain of semenogelin (Zalensky et al., 1993) may suggest that at least some fragments of the SPMI precursor also enter the cell. Moreover, in preliminary experiments, we have observed that both detergent soluble and insoluble fractions of spermatozoa washed twice on Percoll gradients to maximize the removal of seminal plasma proteins and proteins loosely associated with the sperm plasma membrane contained polypeptides recognized by the SPMI antiserum. Interestingly, forms of masses greater than 20 kDa were observed in the insoluble fraction, supporting the notion that SPMI may have penetrated the cells and was thus partially protected from PSA hydrolysis.

On the other hand, the observations that the motility of SPMI-immobilized spermatozoa can be recovered by washing the cells, thus removing the extracellular components, and that prostatic extracellular proteases inactivate SPMI and allow recovery of sperm motility (Chapter IV) argue in favor of a surface-mediated mechanism of action. Moreover, immunodetection of semenogelin using an antibody generated against a basic semenogelin fragment and using the MHS-5 monoclonal antibody generated against the seminal vesicle-specific antigen, localized antigens on the postacrosomal sheath of the head, the mid-region

and the tail of human spermatozoa (Herr et al., 1986; Lilja et al., 1989). SPMI precursor/semenogelin thus appears to bind to the sperm surface in these regions, where it acts on sperm motility. Receptor binding proteins are usually present in trace amounts in biological fluids, and sudden differences in their concentrations trigger biological responses. As such, the abundance of SPMI in seminal plasma would tend to argue against such a mechanism.

The gradual decrease in SPMI activity, as the 52 kDa precursor protein is being cleaved indicates that specific domains of the precursor molecule may be involved in maximizing the interaction between the active domain of SPMI precursor/semenogelin and its target site on spermatozoa. This observation shares similarities with findings concerning inhibin-like peptides isolated from human seminal plasma. Three different inhibin-like peptides were originally isolated from semen as factors that could suppress pituitary FSH secretion *in vitro*, and their complete amino acid sequences were elucidated (Li et al., 1985). All have a primary structure corresponding to segments of semenogelin derived between residues 45 and 136 (Lilja et al., 1989); α-inhibin-92/-52 and -31 correspond to residues 45-136, 85-136, and 85-115 respectively. In the present study, SPMI fragments corresponding to α-inhibin-92 and -52 were observed following PSA-mediated degradation of SPMI precursor and happen to correspond to the active domain of SPMI. Interestingly, α-inhibin-92 and α-inhibin-52 were 40- and 3.4-fold more active, respectively, than α-inhibin-31 in inhibiting FSH release *in vitro* (Li et al., 1985). This decrease in activity of inhibin-like peptides as they are fragmented into smaller peptides is reminiscent to the drop of SPMI activity during processing of the 52 kDa SPMI. The affinity of SPMI and inhibin-like peptides for their respective target appears to be greater for larger fragments. The mechanism of action of these peptides on the pituitary

gland has not been well established, but binding of the peptides to human pituitary membranes has been demonstrated (Ramasharma and Li., 1987). Crosslinking experiments using labeled α-inhibin-92 and pituitary membrane revealed the presence of a 90 kDa complex (Ramasharma and Li, 1987). It is therefore tempting to speculate that SPMI could similarly bind to a specific molecule present on spermatozoa.

These various observations do not allow to draw definite conclusions about the target site of SPMI on spermatozoa but they do suggest potential mechanisms of action. By integrating these various observations, I propose that under normal circumstances, SPMI precursor binds to an acceptor site on the flagellum of spermatozoa and immobilizes the cell. As the precursor gets degraded, its affinity for its acceptor site on spermatozoa is reduced and the molecule is released. This causes the progressive increase in motility observed during semen liquefaction. In other cases where a large proportion of spermatozoa remain immotile in spite of normal semen liquefaction and SPMI precursor/semenogelin processing into low mass fragments, a different mechanism may be responsible. In such ejaculates, a large proportion of spermatozoa may have membrane defects that allow SPMI to attain the intracellular space and reach the dynein arms of the axoneme or other target sites, and cause irreversible arrest of motility and associated infertility. In a preliminary study, de Lamirande and Gagnon (1986) demonstrated that extracts from immotile spermatozoa contained motility inhibitors, whereas normal spermatozoa did not. Unfortunately, no biochemical information was available then to draw definite conclusions about the nature of the factors responsible for this phenomenon. The availability of purified SPMI should now allow to perform specific experiments to answer these important interrogations. Labeled SPMI precursor could be cross-linked to spermatozoa to identify the receptor site on spermatozoa. Similarly the labeled precursor

could also be used in gel overlay experiments, where sperm proteins have been separated, to identify potential protein targets. Immunodetection, at the electron microscopic level, of SPMI in control and SPMI-immobilized spermatozoa could also help in locating target sites of SPMI and elucidating the mechanism of action of SPMI on spermatozoa. In addition, further experiments analyzing multiple ejaculates that fail to liquefy might allow to clarify the way in which the precursor, by interacting with such a target site causes the reversible immobilization of spermatozoa. It may also be interesting to try to identify the functions of the various SPMI fragments released by PSA hydrolysis of the precursor.

6.3 SPMI precursor/semenogelin and semen coagulation-liquefaction

In the present study, a sperm immobilizing activity in seminal plasma was associated with the major protein responsible for human semen coagulation, namely semenogelin. Even though this protein and the two semenogelin-related proteins have been well characterized (Lilja and Laurell, 1985; Lilja et al., 1987; Lilja et al., 1989; Lilja and Lundwall, 1992), the mechanism responsible for the formation of the gelatinous network of proteins has not been convincingly elucidated. As discussed in the introduction (Chapter I), some hints concerning the type of interactions existing between coagulum components have been found experimentally. Disulfide bridges seem to play an active role in the coagulation and liquefaction of human semen since the main proteins of coagulated semen are disulfide bonded, and semen liquefaction is enhanced in the presence of the reducing agent β-mercaptoethanol (Chaistitvanich and Boonsaeng, 1983). On the other hand, dithiothreitol has no macroscopic

effect on the coagulum structure suggesting that these disulfide interactions do not constitute the main force holding coagulum proteins together (Lilja and Laurell, 1985). The role of the reducing agents in the acceleration of liquefaction (Chaistitvanich and Boonsaeng, 1983) is thus probably indirect. Reduction of disulfide-linked coagulum proteins may change their three-dimensional structure and increase their sensitivity to proteolytic activities.

The denaturing agent guanidine-HCl (3 M) readily solubilizes semen coagulum, indicating that non-covalent interactions are essential for maintaining the coagulum structure (Lilja and Laurell, 1985). The fact that urea (4 M) also completely solubilized semen coagulum (Chapter III and IV), is consistent with this finding. In addition, while in rodents transglutaminases from the coagulating gland crosslink protein substrates from the seminal vesicle forming the so-called copulatory plug (Williams-Ashman, 1984), no transglutaminase activity has been detected in human semen (Lilja and Laurell, 1985). Moreover, extensive crosslinking of protein substrates would cause a significant decrease in the mobility of coagulum proteins separated by SDS-PAGE, but no such observations were obtained on coagulum proteins (Lilja and Laurell, 1985; Chapter III and chapter IV).

The mechanism by which non-covalent interactions between proteins trigger semen coagulation remains more speculative. Fibronectin originates from seminal vesicle fluid and, like semenogelin and the semenogelin-related-proteins, gets fragmented during semen liquefaction (Lilja et al., 1987). At ejaculation, prostatic components, such as zinc (see section 6.5), may induce a polymerization reaction involving semenogelins and fibronectin by triggering conformational changes in these proteins, thus inducing coagulation (Lilja et al., 1987). Another model proposed that semen coagulation is induced by the formation of complexes of glycoproteins and metal ions mediated by a redox reaction (Polak and Daunter,

1989). However, as shown in the present study, SPMI precursor/semenogelin does not appear to be glycosilated, thus making such a mechanism unlikely.

On the other hand, the results of several studies clearly demonstrate that the phenomenon of semen liquefaction is associated with the proteolytic degradation of the major coagulum proteins (Lilja, 1985; Boonsaeng, 1986; Lilja et al., 1987; McGee and Herr, 1987; Lee et al., 1989; Chapters II and III). PSA appears to be the main enzyme involved in this process (Lilja, 1985; Lilja et al., 1987; McGee and Herr, 1988). However, the role of PSA during semen liquefaction has been questioned, since the concentration of PSA as measured by immunoassay was found to be the same in highly viscous semen and in liquefied semen (Dubé et al., 1989). However, according to the methodology used, this study dealt with semen samples of high viscosity, rather than non-liquefied semen. Hyperviscous semen forms threads of more than 6 cm when forced through the tip of a Pasteur pipette, whereas normal liquefied semen flows drop by drop. On the other hand, non-liquefied semen consists of a semi-solid gelly-like mass that is not uniformly distributed in semen. A specific case of absence of semen liquefaction, where anomalies in PSA activity levels were likely involved, was presented in Chapter III. While little is known about the cause of semen hyperviscosity, non-liquefied semen is associated with the presence of non-degraded form of SPMI precursor/semenogelin (Chapter III). We have also analyzed several hyperviscous semen samples by SDS-PAGE, and found that SPMI precursor/semenogelin had properly been degraded into low mass forms (data not shown). This indicates that the phenomenon of semen hyperviscosity is not associated with incomplete processing of SPMI precursor/semenogelin in semen by PSA.

While PSA appears to be the main SPMI precursor/semenogelin processing enzyme after

ejaculation, additional proteases may also participate in the phenomenon (Chapter II). Candidate enzymes for this role include the hGK-1 gene product closely related to PSA (Paradis et al., 1989) and the recently characterized prostasin, (Yu et al., 1994) both of which are prostatic trypsin-like proteases that correspond well with the prostatic proteolytic activity inhibited by benzamidine (Chapter II). Even though these enzymes could contribute to cleave the SPMI precursor/semenogelin at alternative sites, their effect is likely to be marginal since PSA cleaves purified SPMI precursor/semenogelin in a way that is almost identical to what is observed in whole liquefied semen (Lilja, 1985; Lilja et al., 1987; Chapters IV and V).

6.4 Possible physiological roles for SPMI and the coagulation of human semen.

The phenomenon of semen coagulation occurs in most mammals but the mechanism varies between species. A notable exception to semen coagulation is found in dogs and cats, which interestingly lack seminal vesicles, the main source of coagulating proteins in several species (Mann and Lutwak-Mann, 1981). The physiological role of semen coagulum during the human reproductive process has not been addressed extensively. In some species, the formation of a vaginal plug is essential to prevent the outflow of semen from the female's vagina. It is believed to act as a reservoir and contribute to the gradual release of spermatozoa in species where the female does not possess a long cervix and cervical mucus, and where semen must be deposited directly into the uterus. In rodents, it is also thought to prevent superfecundation by non-dominant males (Aümuller and Seitz, 1990).

Whether the phenomenon of semen coagulation in humans is simply a remnant of our

evolution from other species is not clearly established. While the amino acid sequence of SPMI precursor/semenogelin does not show significant homology to other known proteins, a segment of semenogelin demonstrates limited, but significant, homology with the rat seminal vesicle secretory protein (SVS-II), a known substrate of the transglutaminase that cross-links proteins to form the vaginal plug (data not shown). In addition, the signal peptides of semenogelin and semenogelin-related proteins and the 3'-untranslated regions of their cDNA are very similar to that of the major seminal clotting protein of guinea pig seminal vesicles, GP1 and rat SVS-II protein (Lilja and Lundwall, 1992; Lundwall and Lazure, 1995).

Some interesting findings reported by Cohen (1990) may provide insights into a possible *in vivo* function for human semen coagulation and liquefaction, and by extension, a role for SPMI. This model proposes a "double-sperm-presentation" pattern relying on the observation that following ejaculation, a few hundred "uninvolved" or untrapped spermatozoa would stimulate the cervical area and elicit a massive immune response in the female host. During that phase, spermatozoa trapped in the coagulum would get coated with immunoglobulins. Leukocytosis might contribute to some type of "sperm selection" and the bulk of spermatozoa might be eliminated. Such reactions were shown to occur rapidly after copulation in rabbits (Smallcombe and Tyler, 1980). The immobilization of spermatozoa within the coagulum by the high level of SPMI activity after ejaculation could nicely fit into that model and actively contribute to the phenomenon. Once the immune reaction occurred and liquefaction is completed, some "selected" sperm could then go on to fertilize.

The semi-solid and adherent properties of the human semen coagulum may also contribute to prevent a massive outflow of spermatozoa from the female's reproductive tract, until they are released progressively during liquefaction. In addition, the bulk of seminal plasma is

removed by passage of spermatozoa through the viscous cervical mucus. I therefore propose that sperm immobilization by SPMI in the first few minutes post-ejaculation might have beneficial effects by providing sufficient time for seminal factors, affecting sperm function, to interact with spermatozoa before crossing the cervical mucus. Moreover, temporary immobilization of spermatozoa by SPMI and their physical protection by the coagulum may also provide the necessary time for seminal factors to elicit immunosuppressive reactions in the female reproductive tract before the progressive release of spermatozoa.

6.5 Potential interactions of SPMI and PSA with seminal zinc.

The results of experiments presented in Chapter V have led to postulate that SPMI precursor/semenogelin may be a zinc binding protein. Zinc is an important ion in biological systems. Although it is usually present only in very low concentrations in body fluids, zinc is present in the prostate gland at the highest concentration of any known organ (Lynch et al., 1994), and is secreted in semen at high levels. However, its role in that fluid remains poorly understood. In coagulated semen, most of the seminal zinc is tightly associated with the coagulated protein mass (Frenette et al., 1989; Mandal and Bhattacharyya, 1990), and more specifically with different polypeptides having identical electrophoretic mobility to SPMI precursor/semenogelin (Frenette et al., 1989). Furthermore, as liquefaction progresses, zinc is associated with progressively lower mass polypeptides (< 25 kDa) of electrophoretic mobilities similar to those of the SPMI precursor/semenogelin degradation products. These experimental results thus support the hypothesis that SPMI may be a zinc binding protein. The presence of bound ions on SPMI precursor/semenogelin supports the hypothesis that this protein is affected by this ion. In prostatic secretions, zinc is present at concentrations up to

7 mM (Lynch et al., 1994) a level sufficient to inhibit most of PSA activity (see Chapter V). At ejaculation, SPMI precursor/semenogelin might chelate most of free zinc, an event that would induce a structural change in the precursor molecule, thus facilitating the non-covalent aggregation of SPMI molecules and the formation of insoluble protein complexes. Following this important decrease in free zinc concentration, PSA activity would be restored, allowing gradual SPMI precursor/semenogelin proteolysis to proceed. As the SPMI precursor/semenogelin would be hydrolyzed into smaller polypeptides during semen liquefaction, zinc would be released into solution due to structural changes around the metal-coordinating residues of the protein. Finally, free zinc would inactivate PSA, thus preventing further undesirable proteolysis of important semen components or damage to spermatozoa. While this simple model remains speculative in nature, it is in agreement with the results obtained in the present study and proposes a new physiological role for the high seminal zinc levels.

6.6 Is the synthesis of SPMI precursor/semenogelin androgen dependent?

The overwhelming abundance of SPMI precursor/semenogelin protein in the secretions of the seminal vesicles, a gland whose secretory function is androgen dependent, argues in favor of an androgen regulated synthesis of this protein. In a preliminary study, semenogelin immunoreactivity was observed on the epithelium of seminal vesicles specimens obtained from post-pubertal subjects, but not on those of a new born or a five year old child (Aümuller et al., 1990). In addition, during the collection of seminal vesicle fluid for the present

experiments, we have observed that the seminal vesicle fluid collected from two patients who underwent radical prostatectomy, and had followed a preoperative treatment with anti-androgens, showed a strong decrease in SPMI activity and immunoreactivity (as observed on Western blots) compared with that obtained from untreated patients (unpublished results). Moreover, the reported semenogelin I gene sequence (Ulvsbäck et al., 1992) contains nucleotide sequences that are closely related to a reported androgen-response element consensus (Luke and Coffey, 1994), in both the promoter upstream sequence and in the first intron of the semenogelin I gene (unpublished results). Together, these observations strongly suggest an androgen-dependent synthesis of SPMI precursor/semenogelin in the human seminal vesicles.

6.7 General considerations about SPMI precursor/semenogelin processing.

The type of protein processing uncovered in the present study is reminiscent of other biochemical pathways making use of precursor molecules. The occurrence of precursor molecules in cells and biological fluids is common. Many hormones and neurotransmitters are synthesized as large inactive prohormone precursor molecules that are proteolytically processed at very specific sites to form smaller active peptides (Docherty and Steiner, 1982). However, in contrast to such systems where the precursor is an inactive pro-molecule, the SPMI precursor is in fact more active than its proteolytic fragments with respect to its sperm immobilizing activity. A particularly interesting precursor molecule is the pro-opiomelanocortin protein precursor that includes, in a single precursor molecule, multiple

peptides that have different biological activities. Upon proteolytic processing, this precursor releases active hormones and neurotransmitters such as the polypeptides melanocyte-stimulating hormone, adrenocorticotropic hormone, enkephalins, β-endorphin and β-lipotropin. Similarly, SPMI precursor/semenogelin may, upon hydrolysis, release polypeptides that have various biological activities. This hypothesis is supported by the fact that PSA is a member of the kallikrein family of proteases, known for their growth factors and vasoactive polypeptides processing activities (Clements, 1989). Possible candidate roles for such bioactive peptides released by SPMI precursor/semenogelin hydrolysis include the inhibin-like peptides isolated from seminal plasma that are derived from residues 45 to 136 of semenogelin (Li et al., 1985; Lilja et al., 1989). The fact that a component of the boar SPMI was found to be highly homologous to a protein shown to be involved in sperm-egg binding (Iwamoto et al., 1995) raises the possibility that a fragment derived from the human SPMI precursor processing may also be involved in the interaction of human gametes. In addition, extended forms of thyrotropin-releasing hormone (TRH)-like peptides have been isolated from human seminal plasma (Khan and Smyth, 1993). These peptides correspond to sequences occurring between residues 350-374 of semenogelin and are thus likely derived from it. The N-terminal of one of these peptides occurs at a position along the semenogelin sequence that is consistent with cleavage by PSA at a leucine residue. Obviously, assessment of the physiological significance of all these activities in seminal plasma will require further investigation. However, these observations highlight the possibility that SPMI precursor/semenogelin may be an important precursor molecule that releases multiple polypeptides with various functions.

Conclusion

Overall, the results of the present study have considerably contributed to broaden the knowledge on SPMI and shed light on the processing of its precursor after ejaculation. SPMI precursor appears to be involved in the immobilization of spermatozoa observed in semen shortly after ejaculation, and in the progressive increase in sperm motility as it gets processed by PSA. Abnormal processing of SPMI precursor may result in low sperm motility and infertility. Moreover, the results also demonstrate that PSA is the main protease involved in the extensive processing of SPMI precursor/semenogelin occurring shortly after ejaculation. PSA is highly specific and displays properties that differ from other known proteases. These new findings on the molecular processing of SPMI precursor/semenogelin should help to pave the way for further functional studies on the role of SPMI precursor/semenogelin and its degradation peptides. Such investigations are necessary to identify precisely the site of action and the mechanism by which SPMI affects sperm motility. The fact that multiple and very different activities can be associated with the same precursor molecule and its degradation products is of significant biological interest. An attempt to integrate these functions and to uncover the nature of the interactions and mechanism of action of these polypeptides during the reproductive process would constitute a logical follow-up to the present study. Since the cDNA for semenogelin has been cloned, mutation and deletion studies followed by expression of the protein in a suitable experimental model could be attempted to answer these interrogations. Such studies will possibly enable better understanding of the modulation of sperm function by seminal plasma components, and consequently lead to novel approaches to the diagnosis and treatment of infertility.

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Claim of original contribution

The following items constitute genuine novel contribution:

1) Identification of an SPMI precursor form in seminal vesicle fluid, and the analysis of its processing in semen after ejaculation.

2) Association of SPMI activity and antigens with the coagulated semen components and demonstration of their detrimental effects on sperm motility. Association of poor sperm motility with absence of SPMI processing.

3) Development of a purification procedure for SPMI precursor, the characterization of its properties and activity on spermatozoa.

4) Characterization of PSA activity on a physiological substrate. Demonstration of PSA-mediated cleavage of SPMI, characterization of this phenomenon and mapping of the precursor molecule.