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PO Box 117
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+46 46-222 00 00

ANTIMICROBIAL ACTIVITY OF HUMAN SEMINAL PLASMA AND SEMINAL PLASMA PROTEINS

Anneli Edström

Department of Clinical Sciences, Lund
Division of Infection Medicine
Faculty of Medicine
Lund University, Sweden

Doctoral dissertation

With due permission from the Medical Faculty at Lund University this doctoral thesis is to be publicly defended on the 29th of October, 2010, at 9.15 in Belfragesalen. BMC D15, Sölvegatan 19, Lund

Supervisor

Ole E. Sørensen

Faculty opponent

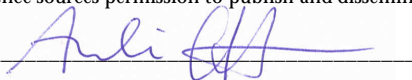
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Abstract <p>Seminal plasma is semen without the spermatozoa. Human seminal plasma is a complex mixture of secretions from the sex accessory glands, mainly the seminal vesicles and the prostate. Seminal plasma has high protein content and it also contains ions, sugars and low molecular weight components.</p> <p>Immediately after ejaculation the semenogelins and fibronectin aggregate and a coagulum forms trapping the spermatozoa. Seminal plasma is then liquefied as the semenogelins are degraded by prostate specific antigen and the spermatozoa are released. We found that the peptides formed when the semenogelins are degraded were responsible for the major bactericidal activity of seminal plasma. The activity of these peptides was strictly zinc-dependent. We also found potent antifungal activity in seminal plasma unleashed by the acid vaginal pH. The antifungal activity was mediated by beta-microseminoprotein (MSP). The activity of MSP was mapped to a region in the C-terminal part of the protein. The antifungal activity was inhibited by calcium binding to MSP at neutral pH but not at the acid vaginal pH explaining the pH-dependent antifungal activity of seminal plasma.</p> <p>This is the first report of antifungal activity of seminal plasma and represents a novel mechanism of regulation of antifungal activity. We also found that the seminal plasma protein, CRISP-3, possibly had an antifungal activity regulated in a pH-dependent manner demonstrating that pH may be an important factor to consider when studying the antimicrobial activity of seminal plasma proteins .</p>		
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Anneli Edström

Department of Clinical Sciences, Lund
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Lund University, Sweden



LUND UNIVERSITY
Faculty of Medicine

Lund 2010

Anneli Edström

Department of Clinical Sciences, Lund
Division of Infection Medicine
Faculty of Medicine
Lund University

Biomedical Center B14
Sölvegatan 19
221 84 Lund
Sweden

E-mail: anneli.edstrom@med.lu.se
Phone: +46 704 882 175
Fax: +46 46 157 756

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ABSTRACT

Seminal plasma is semen without the spermatozoa. Human seminal plasma is a complex mixture of secretions from the sex accessory glands, mainly the seminal vesicles and the prostate. Seminal plasma has high protein content and it also contains ions, sugars and low molecular weight components.

Immediately after ejaculation the semenogelins and fibronectin aggregate and a coagulum forms trapping the spermatozoa. Seminal plasma is then liquefied as the semenogelins are degraded by prostate specific antigen and the spermatozoa are released. We found that the peptides formed when the semenogelins are degraded were responsible for the major bactericidal activity of seminal plasma. The activity of these peptides was strictly zinc-dependent. We also found potent antifungal activity in seminal plasma unleashed by the acid vaginal pH. The antifungal activity was mediated by beta-microseminoprotein (MSP). The activity of MSP was mapped to a region in the C-terminal part of the protein. The antifungal activity was inhibited by calcium binding to MSP at neutral pH but not at the acid vaginal pH explaining the pH-dependent antifungal activity of seminal plasma.

This is the first report of antifungal activity of seminal plasma and represents a novel mechanism of regulation of antifungal activity. We also found that the seminal plasma protein, CRISP-3, possibly had an antifungal activity regulated in a pH-dependent manner demonstrating that pH may be an important factor to consider when studying the antimicrobial activity of seminal plasma proteins .

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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

Paper I

Anneli M.L. Edström, Johan Malm, Birgitta Frohm, Julie A. Martellini, Aleksander Giwercman, Matthias Mörgelin, Alexander M. Cole, Ole E. Sørensen
The major bactericidal activity of human seminal plasma is zinc-dependent and derived from fragmentation of the semenogelins
J Immunol. 2008 Sep 1;181(5):3413-21

Paper II

Anneli M. L. Edström, Victoria Rydengård, Per Fernlund, Matthias Mörgelin, Maria Baumgarten, Alexander M. Cole, Martin Malmsten, Birthe B. Kraglund, Ole E. Sørensen
Beta-microseminoprotein endows seminal plasma with potent calcium-dependent candidacidal activity at vaginal pH
Manuscript

Paper III

Anneli M. L. Edström, Freddi Andersson, Lene Udby, Niels Borregaard, Ole E. Sørensen
Cysteine-rich secretory protein 3 (CRISP-3) and CRISP-3-derived peptides display antifungal activity against C. albicans at the acidic vaginal pH
Manuscript

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IMMUNE SYSTEM

Humans are surrounded by microbes and some are more likely to cause disease (pathogens) while others are less likely to cause disease (commensals). Before the pathogens can cause disease they must first enter the body. We are protected by **mechanical barriers**, such as our skin and mucosal surfaces. Not only are intact skin and mucosal surfaces highly efficient mechanical barriers, they also contain antimicrobial peptides and other substances that form **chemical barriers**. Antimicrobial peptides are present in the skin [1], the respiratory tract [2], the gastrointestinal tract [3], saliva [4], tears [5] and vaginal secretions [6]. Some sites are also protected by a lower pH and a commensal flora, that compete against the pathogens for space and nutrients, as well as producing antimicrobial substances.

Following invasion, we are protected from invading microbes by the immune system. The immune system is a complex system with many components [7]. In order to target the pathogen and not our own cells the immune system must be able to discriminate between self and non-self structures. The immune system is divided into the innate and adaptive immune system (the mechanical and chemical barriers against microbes are viewed as part of the innate immune system) (table 1). The innate immune responses are immediate and non-specific while the adaptive immune responses depend on highly specific interactions and the response to a previously encountered pathogen is faster due to an immunologic memory (which is why vaccines can prevent disease). The innate immune system contains pattern recognition receptors (PRRs) that are encoded in the germline and are present on or in many cell types and recognize patterns unique to microbes (PAMPs – pathogen associated molecular patterns). An example of PRRs are the TLRs (Toll like receptors). So far 10 different TLRs have been identified in humans and these receptors recognize PAMPs such as proteins, lipoproteins, lipids and nucleic acids derived from a wide range of microbes (bacteria, viruses, fungi and parasites) [8].

The receptors of the adaptive immune system are the T-cell receptors (TCR) on the surface of T-cells and the B-cell receptors, also called immunoglobulins (Ig), which can be present both on the B-cell surface and soluble as antibodies in plasma. Both the TCR and the immunoglobulins are encoded in genes with segments that can be rearranged to form highly variable receptors, thereby producing receptors capable of recognizing both self and non-self structures (antigens). In order to remove the receptors capable of recognizing self antigen there are selection steps where these receptors are removed. Sometimes this selection fails and autoimmune diseases can

then arise. T-cells can be cytotoxic and kill infected cells, but they can also be helper T-cells and provide help to B-cells so they can produce antibodies (IgG, IgA, IgM, IgE and IgD). The antibodies can recognize invading pathogens and mark them for destruction by binding to them (opsonization). Opsonized microbes as well as antibody-antigen complex can activate the **complement system**. The complement system is part of the innate immunity and made up of plasma proteins, which when activated, can start a cascade of proteolytic cleavage and formation of active peptides from inactive proteins that fight the infection by opsonizing the pathogen, acting as chemokines, acting as antimicrobial peptides and forming a membrane attack complex (MAC) which forms pores in bacterial membrane and thereby kills bacteria. Effector cells of the innate immune system are neutrophils, monocytes, macrophages, natural killer (NK) cells, eosinophils, basophils and mast cells. **Macrophages** are tissue resident phagocytes (differentiated from **monocytes** in blood) that ingest invading pathogens and secrete cytokines and chemokines that recruit neutrophils from the blood. **Neutrophils** are efficient phagocytes, especially if the pathogen is opsonized, and contain different granules [9] with antimicrobial peptides (AMPs) and other molecules important for eliminating microbes. Neutrophils represent the most numerous leukocytes in the circulation and they are the first effector cells to be recruited to the infection site. Neutrophils eliminate extracellular pathogens by phagocytosis, formation of extracellular NETs (neutrophil extracellular traps), and secretion of antimicrobial molecules. Intracellular pathogens are targeted by NK cells. **NK cells** can detect intracellular pathogens by the lack of certain receptors on the cell surface of the infected cell and can then kill the cell by inducing lysis. **Eosinophils, basophils** and **mast cells** are important for the defense against parasites and are also involved in allergic reactions.

The presence of microbes is not required for an innate immune response. The innate immune response can be triggered in situations where the risk of infection is increased, such as skin injury and sexual intercourse. Sterile wounding of skin causes an inflammatory response with upregulation of antimicrobial peptides and production of IL-8 which acts as a chemoattractant for neutrophils recruiting them to the site of injury [10]. However, the very first response after an injury is exudation of plasma and activation of the complement system, and the contact system leading to activation of the coagulation system. Proteolytic cleavage of proteins in these systems generate antimicrobial peptides [11-14] and other proinflammatory substances. Additional antimicrobial peptides are released by activated platelets [15]. This takes place regardless of if microbes are present or not.

Sexual intercourse can cause tissue damage and lesions and since there is a high microbial load in the urogenital tract this increases the risk of infection. It is therefore important to have immediate protection to minimize the risk of infection. In the female reproductive tract, the vagina and cervix can mount an immune response. When semen is introduced into the vagina it stimulates the migration of leukocytes into the vagina and cervix [16]. The neutrophils may migrate into the cervical mucus, but they will only bind to and ingest spermatozoa if there are both anti-spermatozoa antibodies and complement present [17]. This can happen if the female has become immunized against spermatozoa antigens, but the evidence indicates that the leukocyte invasion is not aimed at the spermatozoa but instead the leukocytes are there to protect against microbes that enter the vagina during coitus [18]. Semen has a potent pro-coagulant activity and this could induce rapid coagulation at lesion sites thereby preventing spermatozoa, seminal plasma components and microbes from entering the blood stream and reduce the risk of generating anti-spermatozoa antibodies and infection [19]. Furthermore, both vaginal fluid and seminal plasma contains antimicrobial peptides able to kill invading microbes.

	Innate immunity	Adaptive immunity
Presence	In all species	In higher vertebrates
Components	AMPs, complement, phagocytes, NK cells, epithelial cells, TLRs*	Lymphocytes (T- and B-cells), antibodies
Specificity	Recognizes a wide variety of fixed PAMPS**	Highly specific
Response	Fast every time	Slow the first time (days) and faster the second time a pathogen is encountered
Memory and immunity	No	Yes

*TLRs: Toll Like Receptors, **PAMPS: Pathogen Associated Molecular Patterns

Table 1. Overview of the differences between the innate and the adaptive immune system.

ANTIMICROBIAL PEPTIDES

A brief historical view

The presence of antimicrobial components in blood and other bodily fluids, leukocytes and tissues has been known since the end of the 1800s and many were reported in the first half of the 1900s [20].

In the 1950s Hirsch reported the presence of a bactericidal protein in rabbit granulocytes that he called phagocytin [21, 22] and then, in the 1960s, Zeya and Spitznagel [23-25] found cationic proteins in leukocytes, isolated from rabbit and guinea pig, to be antimicrobial.

At that time the known mechanism of killing microbes phagocytosed by leukocytes was dependent on oxidative burst and formation of superoxide anions. The neutrophils of patients suffering from CGD (chronic granulomatous disease) are not capable of producing an oxidative burst, due to a defect in the NADPH oxidase, and have only impaired ability to kill many ingested microbes. There must therefore be a second mechanism of killing microbes in granulocytes. The second mechanism was investigated and Lehrer described an antifungal (candidacidal) mechanism in human neutrophils [26] and in monocytes [27] that was dependent on cationic proteins. Cationic proteins from human granulocytes were found to have antibacterial activity as well [28].

Insects were also of interest in this research area since they have no adaptive immunity but still have a fully functional immune system. Boman and coworkers purified and characterized antimicrobial peptides from the hemolymph of immunized pupae of *Hyalophora cecropia*, the giant silk moth, and called them cecropins [29, 30].

Shortly after antimicrobial cationic proteins from rabbit alveolar macrophages were discovered, purified and named MCP-1 and 2 [31, 32]. Another milestone was the discovery of defensins with the isolation and purification of human neutrophil peptides (HNP) 1-3 from human neutrophil granules [33]. Antimicrobial peptides were also found in other tissues. Zasloff noticed that frogs (of the species *Xenopus laevis*) that had undergone non-sterile surgery and had afterwards been placed in a non-sterile environment healed their skin wounds without signs of infection or inflammation. From their skin he isolated antimicrobial peptides and called them

magainins (after magain, the Hebrew word for shield) [34]. More recently psoriatic scales in human skin has been a prominent source for isolation and identification of antimicrobial peptides [35].

After these important discoveries the research field of antimicrobial peptides greatly expanded and today more than 1500 antimicrobial peptides can be found in the Antimicrobial Peptide Database (<http://aps.unmc.edu/AP/main.php>).

Antimicrobial peptides

AMPs are usually defined as proteins with less than 100 amino acids that have broad-spectrum antimicrobial activity (mostly antibacterial, antifungal and anti-viral, but some also antiparasitic [36]). Most of the AMPs are cationic. This means that they have a positive net charge, often +2 to +9 at physiological pH, due to an excess of positively charged amino acid residues (arginine and lysine and under acidic conditions also histidine). Generally 50% or more of the amino acids are hydrophobic and AMPs often adopt an amphipathic structure when in contact with lipid surfaces. This means that there is a polar (hydrophilic) and a non-polar (hydrophobic) part of the peptide that are separated spatially. Many AMPs are salt-sensitive and require a low salt concentration environment for optimal activity. The salt-sensitivity is proposed to be due to instability of the alpha helix and strong electrostatic interactions in high salt conditions [37].

AMPs have been found in all species from bacteria [38], to fungi [39], amoebae [40], insect [29], birds [41], amphibians [34], fish [42], reptiles [43], pigs [44], primates [45] and humans [33] as well as plants [46].

AMPs are a very diverse group of peptides and can be broadly divided into a few groups based mostly on their structural characteristics. Often the peptides are unstructured in a hydrophilic environment and these structures are then formed when the peptide comes in contact with lipid surfaces or in a hydrophobic solution.

Amphipathic alpha-helical peptides

Alpha-helical peptides has been found in all species investigated, which mean that they are present in organisms that are evolutionarily distant. They are some of the most well studied and characterized AMPs. These peptides are unstructured when in an aqueous solution but become alpha-helical when in contact with lipid surfaces, such as cell membranes, or in hydrophobic solutions. They have potent and broad

spectrum antimicrobial activity (active against Gram-positive and Gram-negative bacteria, virus, fungi and protozoa), but they can also have cytotoxic activity and cause hemolysis of human erythrocytes. Examples of this kind of AMPs are human LL-37 [47], amphibian magainins [34], insect cecropin [29], murine CRAMP [48] and bovine seminalplasmin (caltrin) [49].

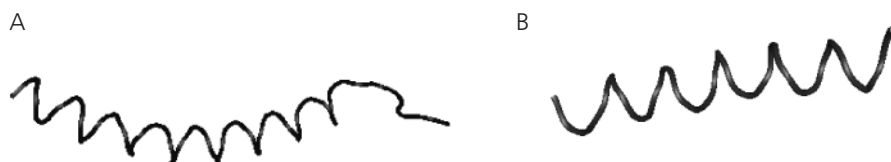


Figure 1.
Solution structure of the alpha-helical peptides LL-37 [50] (A) and magainin [51] (B)

Beta-strands stabilized by disulfide bonds

The AMPs in this group often have antiparallel beta-strands and disulfide bonds that stabilize the structure. Peptides stabilized by one disulfide bond include brevinins [52] from frogs and dodecapeptide from bovine neutrophils [53]. Protegrins from pigs and tachyplesins from horseshoe crabs [54] are examples of AMPs with two disulfide bonds. Defensins that are present in several cells and tissues in several species from insect to humans and also in plants are examples of AMPs that contain six cysteines and three disulfide bonds (reviewed by Ganz [55-57]). The alpha- and beta-defensins differ in the spacing of the cysteines and the pairing of the cysteines forming the disulfide bonds and the theta-defensin (RTD-1) from the rhesus monkey is a cyclic peptide [58]. Peptides that are stabilized by four disulfide bonds include drosomycin from drosophila [59] and human hepcidin [60].

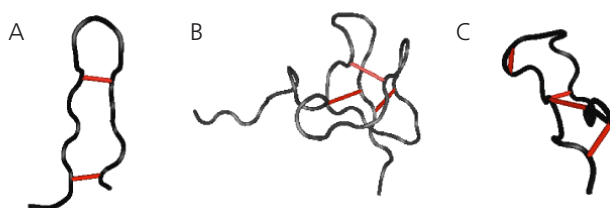


Figure 2.
Solution structure of the beta-strand peptides tachyplesin [61] (A), HBD-3 (human beta defensin 3) [62] (B) and hepcidin [63] (C).

Peptides enriched in specific amino acids

The AMPs in this group are enriched in one or two amino acids and they are variable in structure. Belonging to this group are peptides enriched in proline and arginine residues, such as porcine PR-39 [64] and bovine Bac5 and Bac7 [65], histidine-rich proteins, such as human histatins [66, 67] and peptides with a higher than average content of tryptophan, such as bovine indolicidin [68].



Figure 3.
Solution structure of indolicidin [69], a peptide enriched in tryptophan.

Peptide fragments derived from larger proteins

Antimicrobial peptides that are fragments derived from larger proteins can have similar structure and function to any of the other groups of AMPs. Examples of this group are lactoferricin generated from lactoferrin [70], C3a derived from the complement factor C3 [11] and the semenogelin derived peptides that are responsible for the major antibacterial activity in human seminal plasma [71] (paper I).



Figure 4.
Solution structure of lactoferricin [72], a peptide derived from lactoferrin.

Anionic peptides

The anionic (negatively charged) antimicrobial peptides are small peptides that are produced in mM concentrations and often require zinc as a cofactor for antimicrobial activity (active against both Gram-positive and Gram-negative bacteria). An example of this type of peptide is dermcidin, which is found in human sweat [73].



Figure 5.
Solution structure of the anionic peptide dermcidin (Jung, Yang, Kim MMDB ID 80361)

AMP mode of action

Antibacterial peptides

AMPs can kill microbes by membrane active or non-membrane active mechanisms [74]. All peptides have to first interact with the cell membrane regardless of if they permeabilize it or just translocate across it to reach the cytoplasm and their intracellular target. The initial interaction between the peptide and the target bacteria is thought to be electrostatic bonding between the positively charged peptide and the negatively charged molecules on the outer bacterial surface (such as lipoteichoic acids on Gram-positive bacteria and lipopolysaccharides on Gram-negative bacteria). The peptides make their way to the bacterial cell membrane and enter the interfacial region of the membrane (the interface between the hydrophobic and the hydrophilic portions of the membrane) through electrostatic and hydrophobic interactions. Bacterial membranes are more sensitive to cationic peptides than eukaryotic membranes because they have a higher proportion of negatively charged lipids than eukaryotic membranes.

Several models to explain how AMPs insert into the cell membrane to form pores that cause permeabilization have been proposed, and these models are called the aggregate model, the toroidal pore model, the barrel-stave model and the carpet model (figure 6). The amphipathicity of AMPs is very important in all the models as it allows the hydrophobic regions to interact with the lipid part of the membrane and the hydrophilic regions to interact with the phospholipid head groups or the lumen of the pore.

In the toroidal pore model and the barrel-stave model actual organized pores are formed in the cell membrane, and in the aggregate model more informal channels of varying sizes are formed, whereas in the carpet model patches of the membrane are broken up into micelles (reviewed [74, 75]).

The peptides that kill bacteria without disrupting the cell membrane translocate across the membrane to their various intracellular targets. The modes of action include inhibition of cell wall synthesis, protein synthesis, nucleic acid synthesis and enzymatic activity [74].

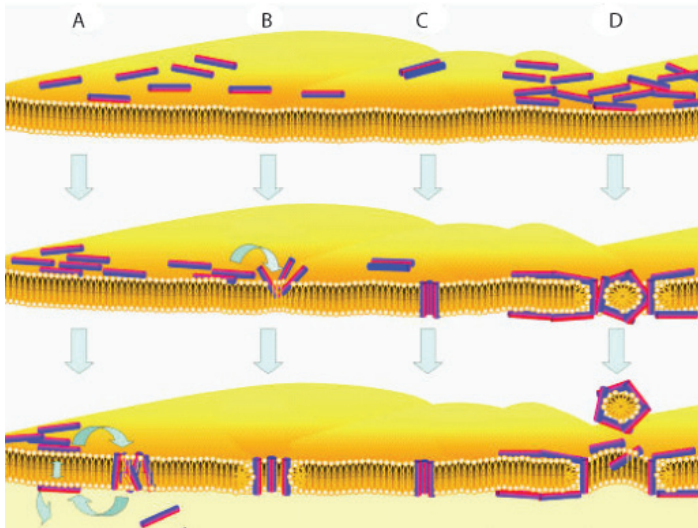


Figure 6. Schematic drawing of the mechanisms of action of anti-bacterial peptides. The bacterial membrane is shown as a yellow lipid bilayer and the cylinders are peptides with red hydrophilic and blue hydrophobic regions. Models to explain mechanisms of membrane permeabilization are the aggregate model (A), the toroidal pore model (B), the barrel-stave model (C) and the carpet model (D). Figure modified from Jenssen *et al.* 2006 [75].

Antifungal peptides

Since fungi are eukaryotic cells their cell membranes are much more like human cell membrane than bacterial cell membranes. However, AMPs can still cause permeabilization of fungal cell membrane but there are no models to explain the mechanism. Not all antifungal peptides target the cell membrane. *C. albicans* mitochondria are the targets of histatins from human saliva [76, 77] and the small cysteine-rich plant peptide Pn-AMP1 causes depolymerization of the actin cytoskeleton of *S. cerevisiae* [78].

Antiviral peptides

Antiviral activity is found in a variety of AMPs and the sensitive viruses are primarily enveloped RNA and DNA viruses. The modes of action often appear to be inhibition of viral entry into the cell by different mechanisms or direct interaction with the envelope (reviewed [75]).

Host defense peptides

Lately it has become apparent that AMPs have more functions than the direct killing of microbes. Accordingly, AMPs are sometimes referred to as host defense peptides. These peptides can interact with cells of the innate immune system, such as neutrophils, monocytes, macrophages and epithelial cells. This can cause increased production of chemokines and cytokines that promote leukocyte recruitment to the site of infection, altered gene expression and induction of cell differentiation. The peptides can also activate or block the TLRs (toll like receptors). This immunomodulation results in innate immune responses that promote wound healing, initiate of adaptive immune responses, limit potentially harmful effects of inflammation and promote clearance of the infection (reviewed [79-82])

MALE UROGENITAL TRACT

The organs of the male reproductive system consists of the testes, epididymis, accessory sex glands (seminal vesicles, prostate, bulbourethral glands (Cowper's glands) and Littre glands), and a system of ducts and structures (figure 7).

The testis produce spermatozoa that are then stored in the epididymis and upon ejaculation the spermatozoa are mixed with the secretions from the accessory sex glands to form the ejaculate.

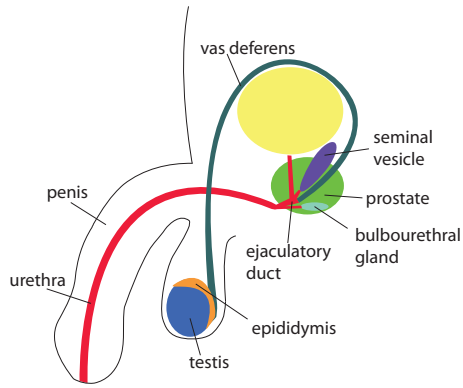


Figure 7.
Schematic view of the male urogenital tract.

EJACULATION

Peristaltic contractions of the smooth muscles in the ducts of the testis, epididymis, ductus deferens, the walls of the prostate and the seminal vesicles propel the spermatozoa and seminal fluid into the urethra and these contractions are then combined with rhythmic contractions of skeletal muscles in the perineum and at the base of the penis, and the semen is ejaculated. The fluids from the different glands are secreted in a specific order (figure 8).

1. Fluids from the bulbourethral (Cowper's) glands and the Littre glands prime the urethra for the passage of sperm. These fluids are slightly alkaline and neutralize any acidic urine residue present in the urethra and they also act as a lubricant. This fluid mix is called the pre-ejaculate.
2. Fluids from the prostate (along with some sperm cells) come into the urethra.
3. The sperm cells mixed with fluids from the epididymis and the ducts come through the vas deferens into the urethra and start to mix with the prostatic fluid.
4. Lastly the fluid from the seminal vesicles, which makes up most of the volume of the ejaculate, is deposited into the urethra and the semen is ejaculated.

After ejaculation the semen will coagulate (because of aggregation of the semenogelins and fibronectin) and trap the spermatozoa. Enzymes (especially PSA, prostate specific antigen) then begin to degrade the semenogelins and the coagulum is liquefied and the spermatozoa are released.

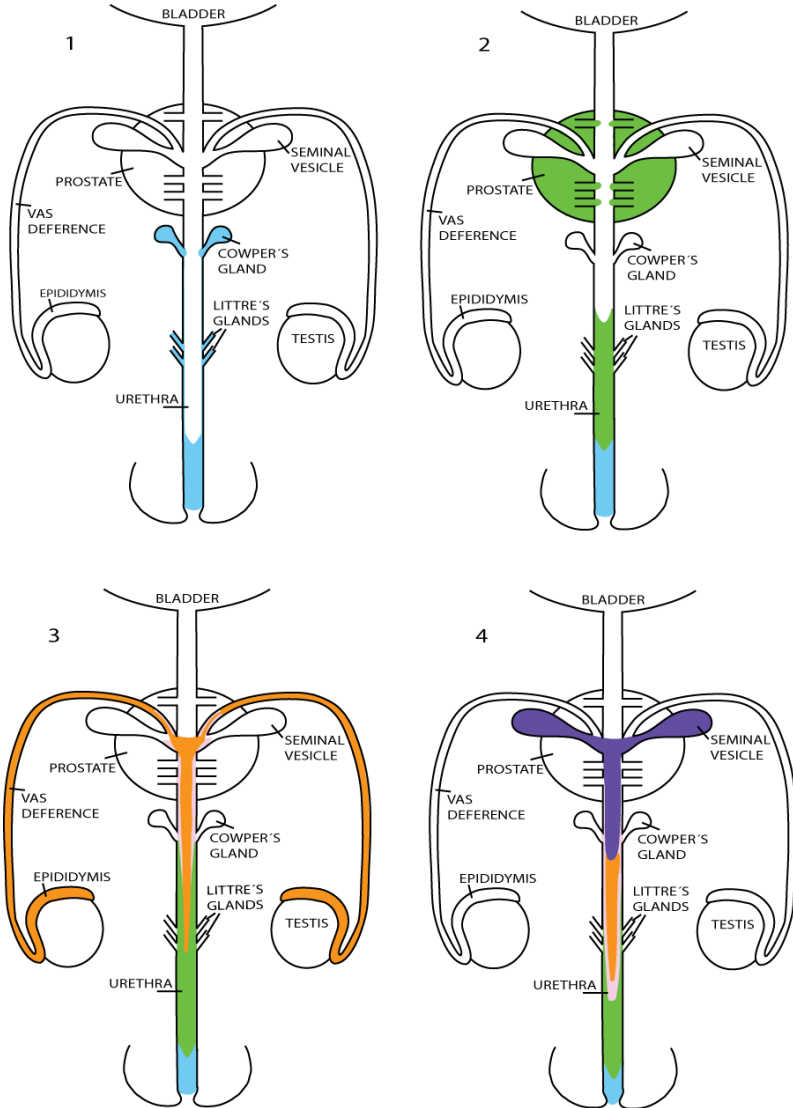


Figure 8. Schematic view of the male urogenital tract depicting the order in which the fluids from the different glands form the ejaculate

SEMINAL PLASMA

Seminal plasma is basically semen without the spermatozoa and an average of about 3.5 ml is released upon ejaculation. It has a whitish color, a distinctive smell and is very viscous. Seminal plasma is a slightly alkaline fluid (pH 7.5-8) [83] made by the seminal vesicles, prostate and the bulbourethral (Cowper's) glands with some contribution from the epididymis, the ducts and the Littre glands (table 2). The **seminal vesicles** make the largest contribution to the seminal plasma of all the contributing glands, about 65-75% of the fluid. Among the components originating in the seminal vesicles are proteins, such as semenogelin I and II [84], lactoferrin [85], fibronectin [86] and progastricsin [87], and low molecular weight components, such as fructose (energy for the sperm cells), prostaglandins (immune suppressant in the female tract), and citrate (involved in the buffering capacity of seminal plasma).

The **prostate** is the second biggest contributor of fluids to the seminal plasma, 25-30% of the total volume. From the prostate comes ions, such as zinc, calcium and magnesium, and proteins, such as prostatic acid phosphatase, beta-microseminoprotein, prostate specific antigen (PSA) [88], lactoferrin [85] and progastricsin [87]. Most of the remaining fluids come from the **bulbourethral glands (Cowper's glands)** and consist of the pre-ejaculate, an alkaline mucus-like fluid that neutralize the acidity of any urine residue in the urethra and also provides lubrication. There is also some small contribution from the **epididymis** containing transferrin [89] and hCAP-18 [90]. Seminal plasma also contains albumin [91] and immunoglobulins [92] that are probably transudated from the intercellular fluid.

Seminal vesicles	65-75%	Semenogelin I and II, fructose, prostaglandins, citrate, lactoferrin, fibronectin
Prostate	25-30%	Prostatic specific antigen (PSA), prostatic acid phosphatase (PAP), beta-microseminoprotein (MSP), progastricsin, zinc, calcium, magnesium, prostasomes
Bulbourethral glands (Cowper's glands)	>1%	Mucus, glycoprotein, galactose, pre-ejaculate

Table 2.
The contributions made by the different glands to seminal plasma.

Seminal plasma is a complex mixture of proteins, low molecular weight components, ions and sugars and the main components and their concentrations are listed in table 3.

Component	Measured values from literature	Mean average values from literature
Total protein	3700-7460 mg/100 ml	5040 mg/100 ml
Albumin	1100-2000 mg/100 ml	1550 mg/100 ml
Glucose	4.3-295 mg/100 ml	102 mg/100 ml
Fructose	136-628 mg/100 ml	272 mg/100 ml
Citrate	304-751 mg/100 ml	528 mg/100 ml
Urea	-	45 mg/100 ml
Lactic acid	-	62 mg/100 ml
Ca	13.7-53.3 mg/100 ml	27.6 mg/100 ml
Ca ²⁺	0.68-1.5 mg/100 ml	0.95 mg/ 100 ml
Mg	5.44-31.8 mg/100 ml	11 mg/100 ml
Zn	6.78-69.29 mg/100 ml	16.5 mg/100 ml
Na	235.6-512 mg/100 ml	300 mg/100 ml
Cl	112-158 mg/100 ml	142 mg/100 ml
K	50-247.7 mg/100 ml	109 mg/100 ml
Total volume	2.3-4.99 ml	3.4 ml
pH	7.26-8.47	7.7
Osmolarity	254-422.7 mosm	354 mosm

Table 3.
Important components of seminal plasma [83].

Coagulation and liquefaction

Directly after ejaculation the seminal plasma begins to coagulate, presumably to prevent loss of semen out of the vagina. The semenogelins and fibronectin aggregate and form a clot [86, 93, 94]. This coagulum traps the spermatozoa and maintains a neutral pH in their vicinity. This is important because the spermatozoa begin to be immobilized when pH drops below 6 [95]. The clot then liquefies within 20 minu-

tes after ejaculation as the semenogelins and fibronectin are degraded into smaller fragments by prostate specific antigen (PSA) [96-98]. As the coagulum liquefies, the spermatozoa are released and well placed to proceed into the cervical mucus, which has a neutral pH. The remaining seminal plasma maintains a neutral pH for about 4 h [99] before the pH is lowered to the vaginal pH (about pH 4). At low pH the progastricsin in seminal plasma will be cleaved into the active enzyme gastricsin and start to degrade various proteins. For example, the human cathelicidin hCAP-18 will be degraded into the antimicrobial peptide ALL-38 [100]. Some of the seminal plasma proteins are quickly degraded by gastricsin, such as PSA and prostatic acid phosphatase, whereas others are degraded more slowly, such as albumin and lactoferrin, and some are not so sensitive to proteolytic degradation by gastricsin, such as beta-microseminoprotein. Possibly, this degradation is a mechanism to decrease the amount of antigenic material in the vagina and prevent the formation of antibodies against seminal plasma proteins and thereby prevent immune infertility [101].

Functions of seminal plasma

Seminal plasma is very important for fertility. Secretions from all glands are necessary for optimal seminal plasma function. Hypofunction of seminal vesicles and prostate is associated with impaired fertility [102-104]. Seminal plasma provides protection, nutrition and components needed for the spermatozoa to mature and be able to fertilize an ovum. The buffering capacity of seminal plasma protects the spermatozoa from the acidic vaginal pH and immunosuppressive substances modulate the immune response in the female reproductive tract. Fructose provides nutrition for the spermatozoa and is important for their survival [105]. The motility of spermatozoa as well as the capacitation and the acrosome reaction, which are needed in order for spermatozoa to fully mature, are influenced by components in seminal plasma [103].

Buffering capacity

The buffering capacity can be measured in the unit slyke, which is the number of micromoles of HCl that must be added to 1 ml of fluid to lower the pH from 7 to 6. Seminal plasma has a much higher buffering capacity than other bodily fluids. The mean buffering capacity of seminal plasma has been reported to be 41.9 slyke [106] while that of blood serum is 23.3 slyke [106], that of saliva is 14.2 slyke [107] and that of tear fluid (calculated by [106] from data by [108]) is only about 7% of that of

seminal plasma. The quantitative role of different components has been investigated and half of the buffering capacity is dependent on proteins and the $\text{HCO}_3^-/\text{CO}_2$ system while that other half is dependent on low molecular weight components [109], probably citrate [110]. The spermatozoa do not contribute to the buffering capacity of seminal plasma [109].

Ions in seminal plasma

Important cations found in seminal plasma include calcium, magnesium, zinc, sodium and potassium ions. Seminal plasma has a high calcium ion buffering capacity and only a small portion, 2-4%, of the calcium is present in ionized form [111, 112]. Citrate is responsible for the calcium ion buffering capacity and is most likely the major regulator of ionized calcium levels in seminal plasma [113, 114]. Calcium may also bind to other proteins and to the surface of spermatozoa. Citrate has a high affinity for calcium, magnesium and zinc, but the concentration of citrate is more than double that of the divalent cations, leaving much of the citrate anionically charged [115]. The concentrations of calcium, zinc and magnesium are correlated [116] as they all originate in the prostate. Zinc is found in a high concentration, about 2 mM, in seminal plasma compared to 10-18 μM in plasma [83]. Zinc is secreted from the prostate in a complex with citrate and after ejaculation half of the zinc is redistributed and bound to proteins, mainly the semenogelins [117, 118]. Zinc ions are antibacterial [119], but there is very little free zinc in seminal plasma. Magnesium, potassium and sodium also tend to form complexes with seminal plasma components. The salt (NaCl) concentration in seminal plasma is lower than that in plasma [83].

Proteins in seminal plasma

Seminal plasma is a protein rich fluid with an average protein concentration of 35-55 g/l [120]. Attempts have been made to identify the protein constituents. For instance, in 2004 Fung *et al.* reported finding over 100 proteins and peptides using gel electrophoresis (1D and 2D) and mass spectrometry (MALDI-TOF-MS or LC-MS/MS) [121]. Lately there have been great technological advances in the field of proteomics and by using more advanced mass spectrometry methods Pilch and Mann reported the identification of 923 proteins in seminal plasma from a single individual [120]. The most abundant proteins are listed in table 4.

Semenogelin I
Semenogelin II
Fibronectin
Lactoferrin
Serum albumin
Laminin

Table 4.
Main proteins found in seminal plasma by Pilch and Mann [120].

Out of all these proteins several have been reported to have antimicrobial activity. The presence of **antimicrobial peptides in human seminal plasma** has been known for a long time. In the 1960s and 70s came reports on the presence of lactoferrin [122] and lysozyme [123], both known antibacterial proteins [124, 125]. Since then several antimicrobial proteins and peptides have been identified (table 5) and their individual physiological importance is difficult to determine. Most of these have been identified as antibacterial, but the cationic peptides in human seminal plasma have been reported to have antiviral activity against HIV virus [126] and in paper II we identify a seminal plasma protein with antifungal activity.

AMPs in seminal plasma	Ref.
Lactoferrin	[85]
Lysozyme	[123]
Semenogelin derived peptides	[71, 127, 128]
MIG/CXCL9	[129]
EPPIN	[130]
HEL-75	[131]
hCAP-18/ALL-38	[90, 100]
Phospholipase A2	[132]
SLPI (Secretory leukocyte protease inhibitor)	[133]
Protein C inhibitor	[134]
Defensins	[135]
Prostasomes	[136]

Table 5.
List of antimicrobial components identified in seminal plasma.

Seminal plasma proteins of special interest in this thesis

In this thesis four seminal plasma proteins are of special interest and these are semenogelin I and II, beta-microseminoprotein and CRISP-3.

Semenogelins

After ejaculation there is a spontaneous coagulation of human seminal plasma. The predominant structural proteins of this coagulum are the major secretory proteins of the seminal vesicle, the semenogelins.

There are two semenogelins, semenogelin I (SgI) and semenogelin II (SgII). These proteins have about 80% identity in primary structure and SgI contains six 60-aa residue regions with internal sequence similarity and SgII contains eight such regions. SgI is a single chain, unglycosylated protein of 439 aa (52 kDa) and SgII has 559 aa [137] and can be both unglycosylated (71 kDa) and glycosylated (76 kDa) [94]. Neither the primary structure nor the repetitive regions are similar to motifs from proteins with known solution structures and hence the solution structure of the semenogelins can not be predicted. The structures have not been solved using NMR or x-ray crystallography. SgI is exclusively expressed in the seminal vesicles whereas SgII is mostly expressed in the seminal vesicles but a minor amount is expressed in the epididymis [138]. The semenogelins are the most abundant proteins in seminal plasma with concentrations of about 50 g/l (1 mM) for SgI and 10 g/l (0.15 mM) for SgII [94]. (table 6)

SgI	SgII
from seminal vesicles unglycosylated 439 aa 52 kDa 1 mM in seminal plasma	from seminal vesicles and epididymis unglycosylated or glycosylated 559 aa 71 kDa or 76 kDa* 0.15 mM in seminal plasma

Table 6.
Properties SgI and SgII.
* glycosylated form

The coagulum that is formed after ejaculation traps the spermatozoa but within 20 minutes the coagulum dissolves and the spermatozoa are released. The liquefaction of the gel occurs due to the enzymatic activity of prostate specific antigen (PSA), a serine protease [96, 98]. PSA degrades the semenogelins (18 cleavage sites in SgI and 16 cleavage sites in SgII) [139] into smaller fragments thereby dissolving the coagulum. There is also some spontaneous degradation of the semenogelins due to their inherent instability. The fragments resulting from the degradation of the semenogelins display antibacterial activity (paper I).

There is a high zinc concentration in seminal plasma (2 mM) [117] but little free zinc ions. The semenogelins are high affinity zinc binders with several zinc binding sites due to their numerous histidine residues (about 7%) [118]. Zinc is an inhibitor of PSA and by binding zinc with a higher affinity than PSA the semenogelins can regulate the enzymatic activity of PSA. Consequently, as long as the semenogelin are able to bind zinc, PSA will be enzymatically active and as the zinc binding ability is lost due to the degradation, zinc will instead bind and inhibit PSA [118].

Beta-microseminoprotein

Beta-microseminoprotein (MSP) is also called PSP94 (prostatic secretory protein of 94 aa) and was previously known as beta-inhibin. It is a cysteine rich protein of 94 aa and 11 kDa. There is about 1 g/l of MSP in human seminal plasma and MSP originates from the prostate. MSP is one of the three most abundant proteins in the prostatic secretion together with PSA and prostatic acid phosphatase [88]. MSP is not a prostate specific protein, although it is present in the highest concentrations there. MSP can also be found in tracheal secretions (about 1/3 of the concentration of that in seminal plasma) and in minor amounts in nasal fluid and gastric juice and also in even lower concentration in other bodily fluids. In fact, MSP has been reported to be associated with mucous secretions [140]. MSP can be found in serum of both men and women in similar concentrations (8 µg/l for men and 6 µg/l for women). In women MSP can be found in follicular fluid at a low concentration (4 µg/l)[141].

MSP is found in several species, such as pig [142], mouse [143], rat [144] and primates [145]. It appears to be a rapidly evolving protein with little identity between the species except for the cysteine residues which are conserved [144].

In 2009 two reports were published on the NMR solution structure of porcine MSP [146] and porcine as well as human MSP [147]. Ghasriani and coworkers reported the solution structures of porcine and human MSP to be very similar, even though the sequence identity is 51%. Their reported structure differed from that reported for porcine MSP by Wang and coworkers (Figure 9). Perhaps further experiments will give more clarity on the subject.

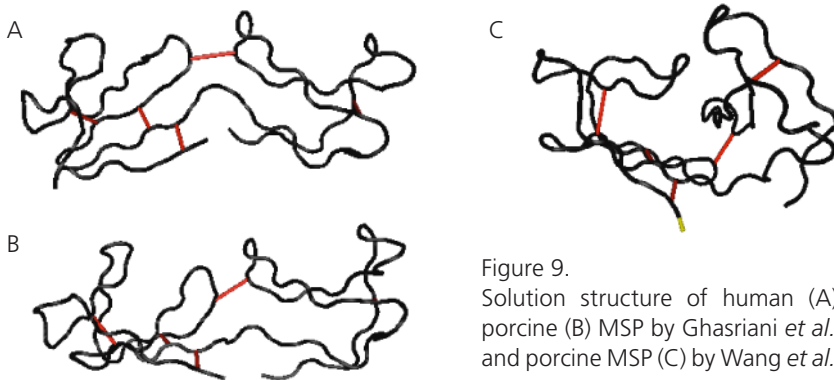


Figure 9.
Solution structure of human (A) and porcine (B) MSP by Ghasriani *et al.* [147] and porcine MSP (C) by Wang *et al.* [146]

MSP was previously known as beta-inhibin because of its reported ability to inhibit the pituitary release of follitropin [148]. However, that report has been questioned as others were not able to verify the findings [149, 150] and the biological function of MSP has yet to be determined. There has been many suggestions as to the function of MSP. MSP has been suggested to decrease tumor growth and hypercalcemia of malignancy in an *in vivo* model of prostate cancer [151], to inhibit sperm motility [152], to bind immunoglobulins in the female reproductive tract [153] and to regulate and induce apoptosis in prostate cancer cells *in vitro* and *in vivo* [154]. Several research groups have investigated the potential clinical use of MSP serum measurements as a biomarker for prostate cancer, but further research is needed [141, 155-158].

MSP binds CRISP-3 (cysteine rich secretory protein 3) in human seminal plasma [159] but this ability has given no clue to its biological function as the function of CRISP-3 is also unclear. MSP also binds to a protein in blood called PSP94-binding protein (PSPBP) [160].

In paper II we propose a biological function for MSP as an antifungal agent activated in the acidic environment of the vagina after sexual intercourse.

CRISP-3

CRISP-3 (cysteine rich secretory protein 3) was so named when it was cloned from testis cDNA [161]. Independently and almost simultaneously the protein was purified from human neutrophils and named SGP28 (specific granule protein of 28 kDa) [162]. CRISP-3 belongs to a family of cysteine rich secretory proteins (CRISPs) and all the CRISPs are about the same size (220-230 aa) and all have 16 cysteine residues that form 8 disulfide bonds. In humans there are three members of the CRISP family, CRISP-1, CRISP-2 and CRISP-3. They display 40-80% sequence identity [161]. Other mammals with described CRISPs are monkeys [163], horses [164], rats [165] and mice [166]. The CRISPs are predominantly found in the male reproductive tract in all these species.

There are also proteins belonging to the CRISP family in venom from several different snake species [167] and a suggested function for some of these proteins is to block K⁺-induced smooth muscle contraction [168]. Also a protein called helothermine from the Mexican bearded lizard is a known toxin that belongs to the CRISP family. This protein can alter ion channels, including voltage-gated K⁺ and Ca²⁺ channels, and ryanodine receptors [169-171].

In humans, CRISP-1 is found in the epididymis and CRISP-2 is a testis-specific protein and both can be found in low concentrations in seminal plasma [161].

CRISP-3 has a wider tissue distribution and can be found in several tissues, such as salivary glands, pancreas, prostate and in less abundance in the epididymis, ovary, thymus and colon [161], and in cells and various secretions, such as in neutrophils (0.18 µg/10⁶ neutrophils), saliva (22 µg/ml), plasma (6 µg/ml), sweat (0.15 µg/ml) and seminal plasma (11 µg/ml) [172].

The function of CRISP-3 has not yet been determined. The localization of CRISP-3 in the reproductive organs might indicate a possible role in reproduction and the presence of CRISP-3 in neutrophils and exocrine secretions point at a possible role in innate immunity. Also, CRISP-3 has sequence similarity with a group of proteins called pathogenesis-related proteins that are thought to play a role in the antimicrobial defense in plants [173].

In addition to binding to beta-microseminoprotein in seminal plasma [159], CRISP-3 also binds to alpha₁beta-glycoprotein in both human [174], cow, horse

and rabbit plasma [175]. The biological function of alpha₁beta-glycoprotein remains to be identified.

In paper III we propose a role of CRISP-3 as an antifungal protein.

THE FEMALE REPRODUCTIVE TRACT

The organs of the female reproductive tract include the ovaries, the fallopian tubes, the uterus, the vagina and the vulva (figure 10).

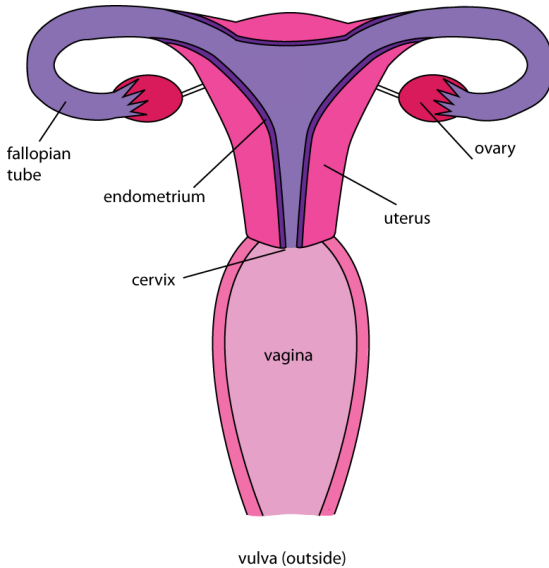


Figure 10.
Schematic view of the female reproductive tract.

In the fertile age the ovaries normally release one oocyte a month. The oocyte travels through the fallopian tubes to the uterus where it leaves the body with the menstrual blood if it is not fertilized. However, if the oocyte meets a sperm cell in the fallopian tube, the fertilized egg will start to divide and attaches to and implants into the uterine wall where it will develop into a fetus.

The uterus, fallopian tubes and ovaries are all sterile sites with no microbial colonization. The vagina however, is heavily colonized, with about 10^7 - 10^8 CFU/g fluid [176]. The healthy vagina of a female in her child-bearing years is mostly colonized by different *Lactobacillus* species. The most common are *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners* [177-180]. It has been reported that other lactic acid producing bacteria can also be the main normal flora in a healthy vagina [181], but *Lactobacillus* species are the most common. The colonizing lactobacilli are beneficial for the host in several ways. By colonizing the epithelial surfaces of the vagina they prevent adhesion of other bacteria and they inhibit the growth of other bacteria by limiting the available nutrients. The lactobacilli are capable of producing hydrogen peroxide, which is antimicrobial, and they can convert host-derived nutrients into organic acids, such as acetic, formic, succinic, propionic, butyric and lactic acid [182]. The lactic acid is particularly important since it lowers the pH of to about 4-5 in a healthy vagina, and it is also antibacterial to invading pathogenic bacteria. In contrast lactic acid is not antimicrobial against *C. albicans* and a few *Lactobacillus* species, all considered part of the normal flora [183]. Apart from the components produced by the normal flora, vaginal fluid is a mix-

ture of fluids including plasma transudate, secretions from the cervical vestibular glands and endometrial and oviductal fluids [184] and has a distinctive and more or less unpleasant odor. Several antimicrobial peptides can be found in vaginal fluid, the majority of them coming from the vaginal epithelial cells, cervical glands and neutrophils. The AMPs include calprotectin, lactoferrin, lysozyme, SLPI, HNP-1,-2, and -3 and HBDs [183]. The cationic polypeptides in vaginal fluid have been reported to have activity against HIV-1 virus in a synergistic fashion [185]. Many components of the innate vaginal defense appear to be under hormonal control and this includes the expression of certain antimicrobial polypeptides, like human defensins 5 and SLPI which are induced by progesterone [186, 187]. Furthermore, immunoglobulins, IgG and IgA, have been found in the cervical mucus and the level of IgA varies during the menstrual cycle, indicating hormonal regulation [188].

While the composition of seminal plasma in the individuals remains pretty constant, the composition of vaginal fluid changes during the menstrual cycle and by sexual arousal. Vaginal fluid is not as protein-rich as seminal plasma nor does it have the same pH-buffering capacity. The pH of vaginal fluid is determined by to what extent the colonizing bacteria produce lactic acid (more lactic acid production means lower pH). The pH of cervical mucus depends on the hormonal environment and this is correlated to the time of the menstrual cycle. Just prior to ovulation there is a predominance of estrogen influencing the cervical glands to make the cervical mucus alkaline (pH of about 8). The mucus also becomes more abundant, watery and will let sperm cells through more easily. In the postovulatory phase there is a predominance of progesterone making the cervical mucus less abundant, thicker and more acidic and not as permissible to sperm cells.

In pregnant women the cervical mucus is thick and forms a large semisolid clot that protects the cervical canal. Not only is this mucus plug a physical barrier between the colonized vagina and the sterile uterus and vulnerable fetus, it also contains several AMPs. These AMPs are SLPI, lysozyme, calprotectin, HNP 1-3, lactoferrin and HBD-1 [189].

In short, the vagina is a harsh acid environment and spermatozoa would have difficulties surviving there long enough to make it into the uterus if not protected by the seminal plasma components. The slightly alkaline seminal plasma uses its buffering capacity to keep the milieu surrounding the spermatozoa at a neutral pH [190] giving the spermatozoa an opportunity to reach the “safe” cervical mucus and move up into the uterus.

VAGINOSIS

Vaginosis is a vaginal infection and there are three major kinds of vaginosis; bacterial vaginosis (BV), candidiasis and trichomoniasis. The symptoms are not the same for the different diseases, but all include abnormal vaginal discharge.

Bacterial vaginosis is, as the name indicates, caused by bacteria. When there is a decline in the normal lactobacillus dominant flora, for instance due to use of antibiotics, the vaginal pH increases and other bacterial strains dominate the flora. These bacterial strains are often a mixture of strains normally found in the gastrointestinal tract, such as the anaerobic bacteria *Mobiluncus*, *Prevotella* and *Peptostreptococcus* and facultative bacteria including *Gardnerella vaginalis*, *Corynebacterium*, *Enterococcus faecalis*, viridians streptococci and coagulase-negative staphylococci [191]. Apart from the change in the colonizing bacterial species, the number of microbes is increased 100-1000 times [192]. Though BV generally is not considered to be a sexually transmitted disease, patients suffering from BV have an increased risk of incurring infections such as HIV-1 and HSV-2 (Herpes simplex) [192], possibly due to the increased vaginal pH and decrease of lactic acid. Furthermore, frequent sexual intercourse is associated with BV [193].

In a healthy vagina AMPs are contributing to the antimicrobial activity [183], but in patients with bacterial vaginosis there are very low levels of AMPs [194]. Following treatment with antibiotics, the level of AMPs is restored to normal, suggesting that BV causes the level of AMPs to drop [194].

Candidiasis is a very common fungal vaginal infection, most often caused by *Candida albicans* [195]. Candidiasis affects 70-75% of all women once during their life time (most frequently during the fertile years). 40-50% of women will experience a recurrence and 5-8% of adult women suffer from recurrent infections, defined as four or more episodes per year [196]. *Candida albicans* can be considered both a pathogen and a commensal and most women carry a low concentration of *C. albicans* at some point [197] without any symptoms of vaginosis. It is not clear why some women suffer from recurrent infections while others are able to be asymptomatic carriers, but it is believed that the innate immune responses play a more significant role than the adaptive immune responses [198]. There are known predisposing factors that increase the risk of candidiasis. Pregnant women have a higher incidence of vaginal colonization and candidiasis [199] possibly due to high concentrations of estrogen which increases the glycogen content in the vagina thereby providing more

nutrition for *Candida*. The same phenomenon can be seen in post menopausal women using estrogen replacement therapy [200]. Genetic factors have been proposed [201-203] as predisposing factors. It is still debated if contraceptives increase the risk of candidiasis as studies have shown conflicting results [196]. Use of antibiotics is considered a risk factor [204], especially in women already colonized by candida [205]. In contrast to BV candidiasis is not associated with frequent sexual intercourse and is seldom transmitted during vaginal intercourse [206].

Trichomoniasis is caused by the protozoan *Trichomonas vaginalis*. Trichomoniasis is a sexually transmitted disease and women that have a more alkaline vaginal pH are more susceptible than women with a healthy low vaginal pH. This is a common protozoan disease in industrialized countries, but it is still uncommon in Sweden.

SEXUALLY TRANSMITTED DISEASES (STDs)

The most common sexually transmitted diseases caused by bacteria are chlamydia (caused by *Chlamydia trachomatis*) and gonorrhea (caused by *Neisseria gonorrhoeae*). Syphilis is also caused by a bacteria (*Treponema pallidum*), but it is no longer a common disease. Chlamydia and gonorrhea can be treated with antibiotics, but are often without symptoms and the infection can therefore go unnoticed and develop into pelvic inflammatory disease (PID) which can cause scarring inside the reproductive organs leading to reduced fertility. Syphilis is a disease with many stages and different symptoms at the different stages. It can be treated with antibiotics if it is discovered in time. Untreated syphilis can be fatal.

Sexually transmitted diseases caused by viruses are hepatitis B (Hepatitis B virus), genital herpes (Herpes simplex virus), HIV (Human immunodeficiency virus) and genital warts/condyloma (Human papilloma virus, HPV). There are no cures for the viral STDs, only drugs that improve the condition of the patients. Genital herpes is normally confined to the genital area, but hepatitis B is a disease that affects the liver and HIV affects certain cells of the immune system. Certain types of HPV cause genital warts while other types cause different kinds of warts and there are also certain types of HPV that can cause cervical cancer.

MATERIAL AND METHODS

Seminal plasma

Semen samples taken at Malmö Fertility Clinic were centrifuged to collect the sperm cells and the remaining supernatants were pooled and frozen. This supernatant is the seminal plasma we have used in our experiments.

Antimicrobial assays

Two forms of antimicrobial assays were our main methods; the gel overlay assay and the colony forming unit (CFU) assay.

Gel overlay assay

This assay is schematically outlined in figure 11.

In a gel overlay assay the antimicrobial activity of individual proteins in a sample containing several different proteins can be determined. The sample is run on an acid-urea (AU) gel, which is a native gel, separating the proteins not only according to size but also according to charge.

The running buffer is 5% acetic acid and the electrodes are reversed resulting in the fastest migration of small cationic proteins. This AU gel is then washed and placed on an underlay agarose containing the preferred microbe. This underlay agarose has a very low concentration of nutrients, so the microbe will survive but not grow.

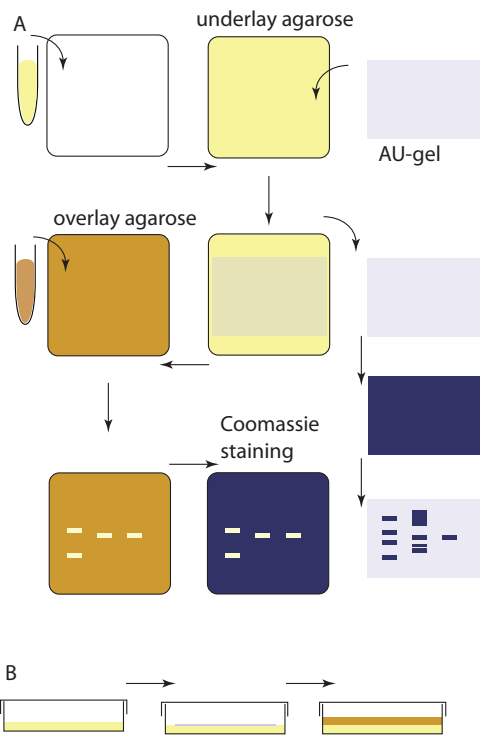


Figure 11. Overview of the gel overlay assay seen from above (A) and from the side (B).

The AU gel on top of the underlay agarose is then incubated and the proteins in the AU gel will migrate into the underlay agarose. The AU gel is then removed and stained and a nutrition-rich overlay agarose is poured on top of the underlay agarose and the plate is incubated over night. The microbes have then grown and form a carpet in the agarose.

If there were antimicrobial proteins in the sample clearing zones lacking microbial growth will be seen and these locations in the agarose can be matched to corresponding bands on the AU gel. For identification of the antimicrobial peptides a duplicate AU gel is run and immediately stained and the bands of interest are cut out and identified.

CFU assay

This assay is schematically outlined in figure 12.

In the CFU assay the total antimicrobial activity of a sample is tested. Basically, your sample is incubated with your microbe of choice for a suitable time and then diluted and plated. After incubation, the colonies on the plates are counted and compared to a buffer control.

Method discussion

Both the CFU assay and the gel overlay assay can be adapted for testing different bacterial and fungal strains and both have advantages and disadvantages. For instance, in contrast to a CFU assay it is not possible to determine if a protein with activity in a gel overlay assay is microbicidal or microbistatic. This is because the protein is present in the agarose gel during the growth of the microbe.

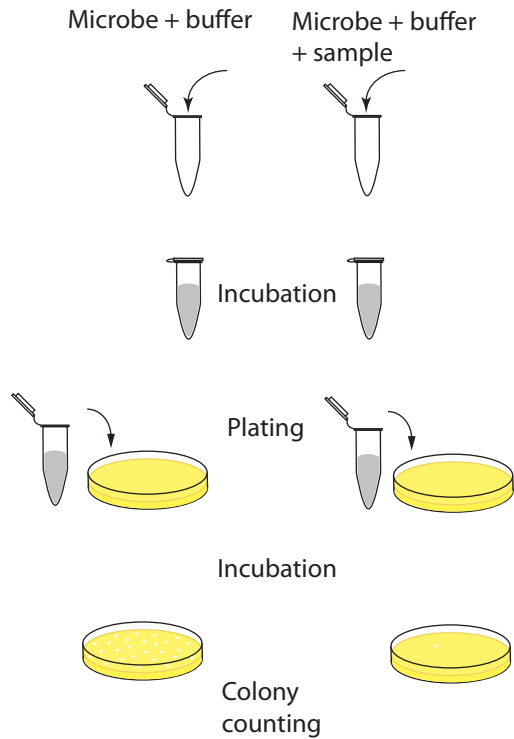


Figure 12. Overview of the CFU assay.

The result “% survival” in the CFU assay must be interpreted as % CFUs and not % surviving microbes. This is especially important when using bacteria that grow in chains or clusters, such as streptococcus and staphylococcus, since it is impossible to determine the number of microbes giving rise to one CFU. The CFU assay is suitable for testing different conditions, such as pH or salt concentration, and addition of substances. However, the buffers or samples used must not contain any other antimicrobial substances than the proteins being tested. The gel overlay assay is relatively insensitive to sample or buffer components (other than proteins) as these substances are removed by electrophoresis.

The gel overlay assay is suitable for testing unstable proteins that need high concentrations of urea to remain stable, such as the semenogelins. However, although the AU-gel is a native gel the proteins might still unfold in a way that renders them inactive or they might be unable to diffuse properly into the agarose gel. Both these situations would cause a false negative result. In the contrary proteins may be unfolded by the electrophoresis and rendered active in a way that would never occur *in vivo*. The conditions in a CFU assay can be more physiological than in a gel overlay assay. It is therefore important that an antimicrobial peptide is active in the CFU assay.

PRESENT INVESTIGATION

The fact that human seminal plasma contains peptides and proteins with antimicrobial activity is old news. Several reports on this subject have been published over the years [85, 90, 100, 123, 126-136, 207]. However, these reports describe that proteins with known antimicrobial activity are present in seminal plasma but do not delineate the importance of these proteins for the antimicrobial activity of whole seminal plasma. We were interested in determining which proteins and peptides that were majorly responsible for the antimicrobial activity of seminal plasma. Most of the identified antimicrobial proteins and peptides in seminal plasma were reported to have antibacterial activity. Accordingly, we began by identifying the components responsible for the overall antibacterial activity of human seminal plasma. This is the topic of paper I.

We then moved on to another group of potential pathogens, the fungi and particularly *C. albicans*. No reports on the antifungal activity of human seminal plasma have been published. Since *Candida* is an important vaginal pathogen, we thought it was interesting to determine if seminal plasma had antifungal activity against *Candida*. We found that seminal plasma did have antifungal activity against *Candida* when subjected to the acid vaginal pH, and this is reported in paper II.

We then started investigating the possible antimicrobial properties of a seminal plasma protein (also found in other bodily fluids) that have some similarities to the antifungal protein we found in seminal plasma, and this is the subject of paper III.

PAPER I

The major bactericidal activity of human seminal plasma is zinc-dependent and derived from fragmentation of the semenogelins

When using “normal” seminal plasma from healthy men we found no antibacterial activity. It is common for antimicrobial peptides to be salt sensitive, the seminal plasma was dialyzed in order to remove the salt. Dialyzed seminal plasma displayed potent bactericidal activity against both Gram-positive and Gram-negative bacterial species. However, no activity was seen against the causative agent of gonorrhea, *N. gonorrhoeae*. When seminal plasma was tested in a gel overlay assay several bands gave rise to clearing zones, indicating that several proteins were responsible for the antibacterial activity. Further analysis of 20 protein bands corresponding to clea-

ring zones identified 15 bands as fragments from semenogelin I or II, 2 bands as SLPI, 2 bands as lactoferrin and one band as phospholipase A2. Semenogelin I and II are the most abundant proteins in seminal plasma and they are responsible for forming the coagulum directly after ejaculation. The coagulum then dissolves as the semenogelins are degraded into fragments by prostate specific antigen (PSA). The semenogelins are produced almost exclusively in the seminal vesicles. We therefore compared normal seminal plasma with seminal plasma from patients with dysfunctional seminal vesicles (with almost no semenogelins) and seminal plasma from vasectomized patients (containing no contributions from the testes and epididymis). Normal seminal plasma and seminal plasma from vasectomized patients displayed the same bactericidal activity whereas seminal plasma from patients with dysfunctional seminal vesicles displayed no antibacterial activity. When the semenogelins were depleted from seminal plasma, the bactericidal activity was lost. We also determined that only the semenogelin fragments generated by PSA cleavage and not the semenogelin holoproteins were antibacterial. The semenogelins bind zinc and we found that the binding of zinc was required for the bactericidal activity. The semenogelin fragments are degraded over time and we found that the loss of semenogelin immunoreactivity corresponded to the loss of bactericidal activity over time.

To summarize; we found that the overall bactericidal activity of human seminal plasma was dependent on fragments derived from semenogelin I and II and that this activity was zinc-dependent and salt-sensitive and the activity was lost as the semenogelin fragments were further degraded.

PAPER II

Beta-microseminoprotein endows seminal plasma with potent calcium-dependent candidacidal activity at vaginal pH

“Normal” seminal plasma, seminal plasma from vasectomized patients (contains no contribution from epididymis and testis) and seminal plasma from patients with dysfunctional seminal vesicles (contains no contribution from the seminal vesicles) all gave rise to a single identical clearing zone when tested in a gel overlay assay against *C. albicans*. The protein responsible for causing this clearing zone was identified as beta-microseminoprotein (MSP). MSP is produced in the prostate and present in seminal plasma in a concentration of around 0.5-1 mg/ml. Recombinant MSP and native MSP purified from seminal plasma also gave rise to clearing zones

identical to the clearing zone produced by seminal plasma when tested in a gel overlay assay. However, when seminal plasma was tested in a CFU assay against *C. albicans* there was no antifungal activity even after dialysis. When the CFU assay was performed under acidic conditions at pH 4 (to mimic the conditions of the gel overlay assay and also the pH of a healthy vagina) both seminal plasma and dialyzed seminal plasma had potent fungicidal activity and could be diluted 1000 times and still retain the activity. This indicated that low pH was important for the fungicidal activity. Furthermore, incubation at low pH endowed seminal plasma with antifungal activity when tested in a CFU assay at neutral pH. Post coital seminal plasma (seminal plasma taken after acidic *in vivo* vaginal incubation for 10 h) also displayed fungicidal activity in a CFU assay at neutral pH and a clearing zone identical to that produced by seminal plasma was found in a gel overlay assay. The fungicidal activity of both MSP and whole seminal plasma was inhibited by calcium. Consequently, EDTA was found to unleash the fungicidal activity of both MSP and seminal plasma. The fungicidal activity was mapped to the C-terminal part of MSP, residues 66-76. This fragment (MSP₆₆₋₇₆) was also sensitive to inhibition by calcium. The inhibition by calcium was due to an interaction between calcium and a glutamic acid residue (E₇₁) in MSP. When the glutamic acid was replaced by a glutamine (MSP_{66-76E/Q}) this peptide was not as sensitive to inhibition by calcium. The calcium inhibition of the fungicidal activity of whole seminal plasma and MSP₆₆₋₇₆ was significantly decreased at the low vaginal pH offering an explanation for the pH-dependent activation of fungicidal activity. A peptide from porcine MSP corresponding to MSP₆₁₋₈₀ was also found to have fungicidal activity indicating that fungicidal activity may be a common feature for members of the beta-microseminoprotein family.

To summarize; we found seminal plasma to have potent fungicidal activity when subjected to the vaginal acidic pH and the protein responsible was beta-microseminoprotein, where the fungicidal activity was located in a segment in the C-terminal part of the protein. Fungicidal activity was also found in the C-terminal part of porcine MSP. The fungicidal activity of seminal plasma was inhibited by calcium in a pH dependent manner, with lower inhibition at pH 4 than at neutral pH. This is the first reported function of MSP as an antimicrobial agent and the first description of a pH-dependent calcium inhibition mechanism for antimicrobial proteins.

PAPER III

Cysteine-rich secretory protein 3 (CRISP-3) and CRISP-3-derived peptides display antifungal activity against C. albicans at the acidic vaginal pH

Cysteine-rich secretory protein 3 (CRISP-3) was purified from neutrophils and found to have antifungal activity against *C. albicans* when tested at the low vaginal pH (pH 4). No antibacterial activity was found when tested at neutral pH and only a little activity when tested at low pH. Overlapping peptides spanning the entire protein were synthesized and tested for antibacterial and antifungal activity. None of the CRISP-3-derived peptides displayed antibacterial activity even at low pH. Depending on the pH and the presence or absence of divalent cations the antifungal activity varied between the CRISP-3-derived peptides. In general peptides encompassing residues 16-35, 31-50, 46-65, 61-80, 186-205, and 201-225 displayed some antifungal activity both at low and neutral pH. The effect of divalent cations varied amongst the peptides but most peptides had enhanced antifungal effect in the presence of EDTA. The active peptides displayed activity in a dose-dependent manner.

To summarize; we found antifungal activity in CRISP-3, a protein of unknown function. This activity was more pronounced at the acidic vaginal pH. Possibly, the activity of the holoproteins was regulated in a calcium-dependent mechanism like MSP. Several parts of CRISP-3 seemed to be important for the observed antifungal activity.

CONCLUSIONS

- Seminal plasma displayed bactericidal activity against *E. coli*, *S. aureus*, *S. pyogenes*, *S. agalactiae* and *E. faecalis* but not *N. gonorrhoeae*.
- Peptides derived from the fragmentation of semenogelin I and II were responsible for the major bactericidal activity of human seminal plasma.
- The bactericidal activity of the semenogelin-derived peptides was strictly zinc-dependent.
- Seminal plasma depleted of semenogelins had no bactericidal activity.
- Seminal plasma lost its bactericidal activity as the semenogelin-derived peptides were further degraded.
- Exposure to low vaginal pH endowed seminal plasma with potent fungicidal activity.
- Seminal plasma displayed fungicidal activity against *C. albicans*, *C. krusei* and *C. parapsilosis* but not *C. glabrata* or *A. fumigatus*.
- The antifungal activity was dependent on beta-microseminoprotein (MSP).
- The activity of MSP was mapped to a region in the C-terminal part of the protein.
- The fungicidal activity of seminal plasma and MSP was inhibited by calcium.
- Calcium bound to a glutamic acid residue in the antifungal region of MSP thereby inhibiting the activity.
- The binding between MSP and calcium was stronger at neutral pH than at pH 4, explaining the pH-dependent fungicidal activity.
- The seminal plasma protein CRISP-3 had a more pronounced fungicidal activity at low pH and could possibly be regulated in the same manner as MSP.
- This is the first reported function of MSP and CRISP-3 as well as the first description of a pH- and calcium-dependent mechanism of inhibition of an fungicidal protein.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Mikroorganismer (bakterier, virus och protozoer) finns överallt i vår omgivning. Vi människor har tio gånger fler bakterier på och i oss än vi har mänskliga celler. De flesta bakterier orsakar sällan eller aldrig sjukdom, men det finns de som ofta orsakar sjukdom och dessa kallas patogener. För att skydda oss från patogener har vi ett komplicerat och välutvecklat immunförsvar som består av många olika celler och molekyler. I alla våra kroppsvätskor finns proteiner och peptider som hjälper till att skydda oss från mikroorganismer. Dessa kroppsegna antibiotika kallas antimikrobiella peptider (AMP).

I denna avhandling har antimikrobiella peptider i sädesvätska undersökts. Sädesvätska är det som blir kvar av sperman när spermerna tagits bort. Sädesvätskan är en blandning av vätskor från olika körtlar, mest kommer ifrån sädesblåsorna och prostata. Vätskorna från de olika körtlarna har olika innehåll, t.ex. proteiner, socker och salter, och när dessa olika vätskor blandas efter utlösningen så koagulerar sädesvätskan och spermerna fångas i ett koagulat. Det är proteinerna semenogelin I (SgI), semenogelin II (SgII) och fibronectin från sädesblåsorna som klumpar ihop sig och bildar koagulatet. Ett enzym från prostatan (prostate specific antigen, PSA) bryter sedan ner proteinerna till mindre peptider och koagulatet löses upp och spermerna släpps fria. Efter utlösningen så hamnar spermerna i en tämligen fientlig miljö i vaginan. En normal vagina är koloniserad av mjölksyraproducerande normalflorabakterier (lactobaciller) och mjölksyran gör att pH i vagina är surt (runt 4). Mjölksyran hjälper till att döda potentiella patogener, men kan också skada spermerna eftersom de inte kan överleva i så lågt pH. Sädesvätskan skyddar spermerna från den sura miljön genom att fungera som en buffert och hålla ett neutralt pH i området runt spermerna så att de kan få en chans att ta sig upp i livmodern, som är steril och har neutralt pH.

Man har länge känt till att det finns flera olika AMP i sädesvätska och att dessa kan döda bakterier och även vissa virus. Det har däremot inte tidigare fastställts om sädesvätska kan döda svamp.

Man kan tänka sig att dessa AMP finns i sädesvätskan för att både skydda spermerna och vaginan från de bakterier som följer med in vid samlag, t.ex. de som finns på huden runt könsorganen, och de bakterier som finns i vaginan och förhindra att de tar sig upp i de övre sterila reproduktionsorganen samt förhindra att de små vävnadsskador som kan uppkomma vid samlag blir infekterade.

Mål med delarbete I

Vi ville ta reda på vilken eller vilka av alla beskrivna AMP i sädesvätska som var viktigast för dess förmåga att döda bakterier.

Sammanfattning av delarbete I

Semenogelin I och II bryts ner till mindre delar (peptider) vid upplösningen av sädesvätskekoagulatet. Eftersom semenogelinerna finns i hög koncentration i sädesvätska blir koncentrationen av dessa peptider också hög. Vi fann att dessa semenogelinpeptider var ansvariga för den största delen av sädesvätskans antibakteriella aktivitet. I sädesvätska finns en hög koncentration av zink och en stor del finns bundet till semenogelinerna. För att semenogelinpeptiderna skulle kunna döda bakterier behövde de ha zink bundet till sig. I takt med att semenogelinpeptiderna bröts helt ner ytterligare så förlorade sädesvätskan sin antibakteriella aktivitet.

Mål med delarbete II

Vi ville fastställa om sädesvätska kan döda svamp, speciellt *C. albicans* som ofta orsakar underlivsinfektioner, och i så fall identifiera proteinet/proteinerna med den antifungala aktiviteten.

Sammanfattning av delarbete II

När obehandlad sädesvätska testades vid neutralt pH saknade den förmåga att döda *C. albicans*. Om sädesvätskan utsattes för pH 4 (samma låga pH som finns i vagina) så kunde den döda *C. albicans* även vid neutralt pH. Något hände i lågt pH som gjorde att sädesvätskan fick antifungal aktivitet. Vi fann att beta-microseminoprotein (MSP) var proteinet som hade antifungal aktivitet. MSP är ett protein med tidigare okänd funktion som bildas i prostatan och finns i sädesvätska i en koncentration av 0.5-1 mg/ml. Genom att dela upp MSP i mindre peptider och testa dessa var för sig kunde vi isolera den del av proteinet där den antifungala aktiviteten fanns. Anledningen till att sädesvätska behövde lågt pH för att döda *C. albicans* var att i neutralt pH band kalcium till MSP och hämmade den antifungala aktiviteten. Vid lågt pH band kalcium inte längre lika bra och MSP fick då antifungal aktivitet.

Mål med delarbete III

Vi ville undersöka om sädesvätskeproteinet CRISP-3 hade någon antibakteriell eller antifungal aktivitet.

Sammanfattning av delarbete III

CRISP-3 är ett protein som bildas i prostatan och bitesticklarna och finns i en koncentration av 11 µg/ml i sädesvätska. CRISP-3 har ännu ingen känd funktion. Våra experiment visade att CRISP-3 inte hade någon antibakteriell aktivitet, men att delar av CRISP-3 hade antifungal aktivitet, speciellt vid pH 4. Detta skulle kunna tyda på att fler protein i sädesvätska är beroende av lågt pH för att ha antimikrobiell aktivitet.

Slutsatser

Att peptider från semenogelinerna var antibakteriella har tidigare beskrivits [127, 128]. I delarbete I drar vi slutsatsen att dessa semenogelinpeptider står för den största delen av den antibakteriella aktiviteten i sädesvätska och att de är beroende av zink för att vara aktiva. I delarbete II rapporterar vi för första gången att sädesvätska har antifungal aktivitet vid lågt pH och att denna aktivitet är beroende av MSP samt att kalcium kan hämma aktiviteten vid neutralt pH men inte vid lågt pH. I delarbete III rapporterar vi för första gången att CRISP-3 och peptider från CRISP-3 har antifungal aktivitet, speciellt vid lågt pH.

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