Characterization of Prostate-Specific Antigen Proteolytic Activity on its Major Physiological Substrate, the Sperm Motility Inhibitor Precursor/Semenogelin I[†]

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ABSTRACT: The protease prostate-specific antigen (PSA) is a marker widely used clinically for monitoring prostatic malignancies. Under normal conditions, this enzyme is mainly involved in the post ejaculation degradation of the major human seminal protein, the seminal plasma motility inhibitor precursor/semenogelin I (SPMIP/SgI), which is the predominant protein component of human semen coagulum. PSA primary structure and activity on synthetic substrates predict a chymotrypsin-like activity whose specificity remains to be established. The present study was aimed at characterizing the proteolytic processing of the SPMIP/SgI by PSA. Purified SPMIP/SgI was incubated with PSA in the presence or absence of protease inhibitors. General serine protease inhibitors, 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 toward SPMIP/SgI. Under identical conditions, other proteins, such as bovine serum albumin, ovalbumin, and casein, were very poor substrates for PSA. Hydrolysis products were separated by reverse-phase high-performance liquid chromatography, assayed for sperm motility inhibitory activity, and analyzed by immunoblotting and mass spectrometry. The region responsible for the sperm motility inhibitory activity and containing an SPMI antiserum epitope was localized to the N-terminal portion of the molecule between residues 85 and 136. On the other hand, a monoclonal antibody against a seminal vesicle-specific antigen (MHS-5) recognized fragments derived from the central part of the SPMIP/SgI (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 SPMIP/SgI in human semen and that this protease manifests unusual specificity with respect to hydrolyzable substrates and sites of hydrolysis.

Human seminal plasma is rich in proteolytic enzymes (Mann & Lutwak-Mann, 1981). One of these proteases, the prostate-specific antigen (PSA),[1] 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 & 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 its major physiological substrate, SPMIP/SgI. Previous results have shown that some of the hydrolysis sites are consistent with a restricted chymotrypsin-like specificity (Robert & Gagnon, 1996).

Following spermatogenesis and maturation during epididymal transit, spermatozoa are stored in the cauda epididymis where they are maintained in an immotile state (Eddy & O'Brien, 1994). Motility is induced at ejaculation when spermatozoa are mixed with secretions from the various male accessory sex glands (Lindholmer, 1974). However, immediately after ejaculation, human spermatozoa remain immotile, trapped in the semen coagulum (Tauber & Zaneveld, 1981) whose major constituent is semenogelin I, a 52 kDa protein expressed exclusively in the seminal vesicles

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[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; ATP, adenosine triphosphate; CAPS, cyclohexylaminopropanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; HBS, HEPES-buffered saline; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid; hGK-1, human glandular kallirein-1; HSS, HEPES saline solution; NPGB, *para*-nitrophenylguanidobenzoate; PSA, prostate-specific antigen; PMSF, phenylmethylsulfonyl fluoride; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPMI, seminal plasma motility inhibitor; SPMIP/SgI, SPMI precursor/semenogelin I; SVSA, seminal vesicle-specific antigen; TBS, TRIS-buffered saline; TFA, trifluoroacetic acid; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone; STI, soybean trypsin inhibitor.

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(Lilja et al., 1989). 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 motility inhibitor (SPMI), was isolated from human seminal plasma (Iwamoto & Gagnon, 1988a). This 19 kDa protein inhibits the motility of intact spermatozoa in a dose-dependent manner (Iwamoto & Gagnon, 1988b). SPMI originates exclusively from the seminal vesicles (Luterman et al., 1991) as a very active higher molecular mass precursor form (Robert & Gagnon, 1994). After ejaculation, the precursor is partially inactivated by transformation into less active, low molecular mass forms by prostatic proteases (Robert & Gagnon, 1994). Recent evidence suggests that absence of normal SPMI precursor processing may result in poor sperm motility and infertility (Robert & Gagnon, 1995).

SPMI precursor has been purified from seminal vesicle fluid and coagulated semen and found to be a potent and reversible inhibitor of sperm motility when tested at levels comparable to those encountered in semen immediately after ejaculation (Robert & Gagnon, 1996). Once processed, the levels of sperm motility inhibitory activity are reduced, allowing the initiation of progressive sperm motility. Biochemical analysis revealed that the SPMI precursor is identical to semenogelin I, the main structural protein of semen coagulum (Lilja et al., 1989; Malm et al., 1996; Robert & Gagnon, 1996). The protein now referred to as SPMI precursor/semenogelin I (SPMIP/SgI) is a basic 49.6 kDa non-glycosylated protein (Malm et al., 1996; Robert & Gagnon, 1996). 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; Malm et al., 1996; Robert & Gagnon, 1996).

In the present study we have investigated the proteolytic processing of SPMIP/SgI by PSA and characterized the activity of this enzyme on its major physiological substrate.

MATERIALS AND METHODS

Materials. Glycine, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid (HEPES), cyclohexylaminopropanesulfonic acid (CAPS), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), benzamidine, and *p*-nitrophenylguanidobenzoate (NPGB) were purchased from ICN Biomedicals (Montréal, QC, Canada); acrylamide, β-mercaptoethanol (β-ME), Coomassie Blue, nitro blue tetrazolium chloride (NBT), and bromochloroindolphosphate (BCIP) were from Bio-Rad (Mississauga, ON, Canada); iodoacetamide, aprotinin, and 1,10-phenanthroline were from Sigma Chemical Co. (St. Louis, MO); adenosine triphosphate (ATP), phenylmethylsulfonyl fluoride (PMSF), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), leupeptin, pepstatin, *N*-α-tosylphenylalanine chloromethyl ketone (TPCK), and tosyllysine chloromethyl ketone (TLCK) 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), Percoll from Pharmacia (Baie d'Urfé, QC, Canada), MHS-5 monoclonal antibody from Humagen Fertility Diagnostics (Charlottesville, VA), and alkaline phosphatase-conjugated goat anti-rabbit and anti-mouse IgG were from Jackson Immunoresearch Laboratories (West Grove, PA). PSA purified from human seminal plasma was obtained commercially from Scripps Laboratories (San Diego, CA). All other chemicals were at least of reagent grade.

Purification of SPMIP/SgI. Seminal vesicle fluid or washed seminal coagulum (Mandal & Battacharyya, 1991) 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. The proteins were reduced by the addition of crystalline DTT to a final concentration of 25 mM and incubated at room temperature for 30 min. 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 min. The proteins were precipitated in 94% ethanol at −70 °C, resuspended in 8 M urea/HSS, and stored at the same temperature until used. Reduced and blocked proteins were loaded on a 5 × 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 0−300 mM NaCl in buffer A at a flow rate of 1 mL/min. Fractions from the S-Sepharose chromatography containing sperm motility inhibitory activity were pooled, made up to 0.1% TFA, and loaded on a Vydac (The Separations Group, Hesperia, CA) semipreparative C_4 protein column (10 × 300 mm, 10 μm 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 min at a flow rate of 3 mL/min. Fractions containing the purified SPMIP/SgI 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 h at 25 °C. Purified SPMIP/SgI 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%, and 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*g* for 30 min, highly motile and morphologically normal spermatozoa from the 65−95% Percoll interface and the 95% Percoll layer were recovered and combined.

Sperm Motility Inhibitory Activity Assay. To identify the polypeptides containing sperm motility inhibitory activity, aliquots of the different eluted fractions were dried in a rotary evaporator and dissolved in 40 μL of a medium whose composition is optimized to allow reactivation of motility for up to 10−15 min (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, 1 mM DTT, and 0.5 mM Mg·ATP). Percoll-washed human spermatozoa (2 μL) were added to the medium, and reactivation of sperm motility was evaluated under a phase-contrast microscope. Fractions in which reactivation of

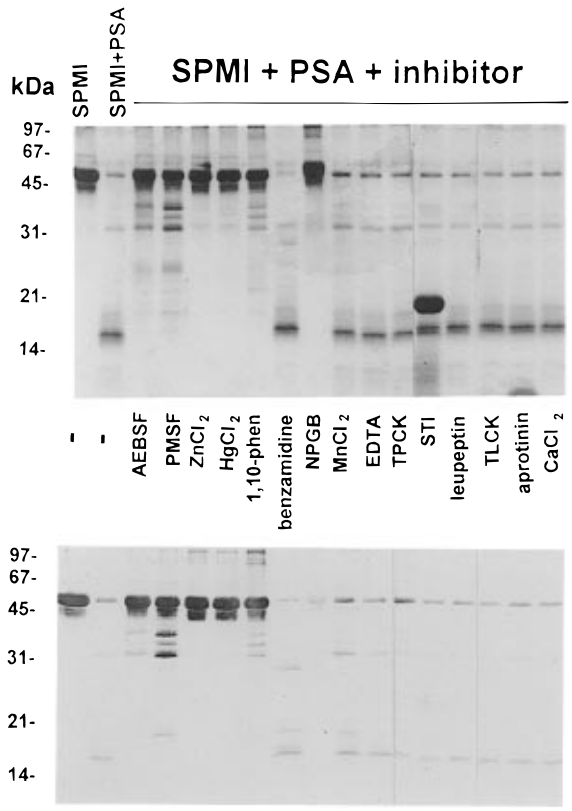

FIGURE 1: Effect of protease inhibitors on PSA hydrolysis of SPMIP/SgI. Various protease inhibitors were pre-incubated with PSA for 18 h. SPMIP/Sg was then added, and hydrolysis was performed for 24 h. The reactions were stopped by the addition of SDS sample buffer and heating to 95 °C for 5 min, and 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 used were as follows: AEBSF and PMSF (5 mM); Zn, Hg, Ca, and Mn (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).

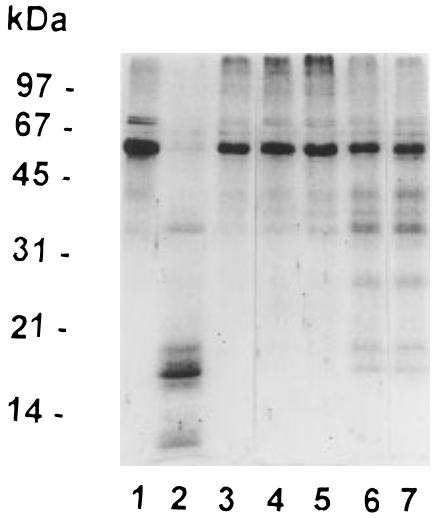

FIGURE 2: Reversal by zinc of 1,10-phenanthroline effect on PSA hydrolysis of SPMIP/SgI. SPMIP/SgI was incubated with PSA in the presence of 1,10-phenantroline (50 mM). After 10 min $ZnCl_2$ was added, and the mixture was incubated for 24 h. The reactions were stopped by the addition of SDS sample buffer and heating to 95 °C for 5 min. Samples were analyzed by SDS−PAGE as described in Materials and Methods. (1) Control SPMIP/SgI. (2) SPMIP/SgI and PSA in the absence of 1,10-phenanthroline. (3) SPMIP/SgI and PSA with 50 mM 1,10-phenanthroline. SPMIP/SgI and PSA with 50 mM 1,10-phenanthroline + (4) 0.1 mM $ZnCl_2$, (5) 0.3 mM $ZnCl_2$, (6) 3 mM $ZnCl_2$, and (7) 10 mM $ZnCl_2$.

motility did not occur were considered to contain sperm motility inhibitory activity.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Proteins 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-mercaptoethanol, and 0.0125% bromophenol blue).

Immunoblotting and Immunodetection. Following electrophoresis, proteins were electrotransferred from slab gels onto Nitroplus nitrocellulose membrane (Micron Separations Inc., Westboro, MA) for 40 min at 70 V in 10 mM CAPS and 10% methanol at pH 9.8. 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% 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 1:400 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 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 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 SPMIP/SgI were separated by SDS−PAGE as described above and transferred onto Immobilon-P membrane (Millipore, Bedford, MA) for 40 min 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.

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 1 μL/min. The system's 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.

Identification of SPMIP/SgI Peptides. The complete semenogelin I 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

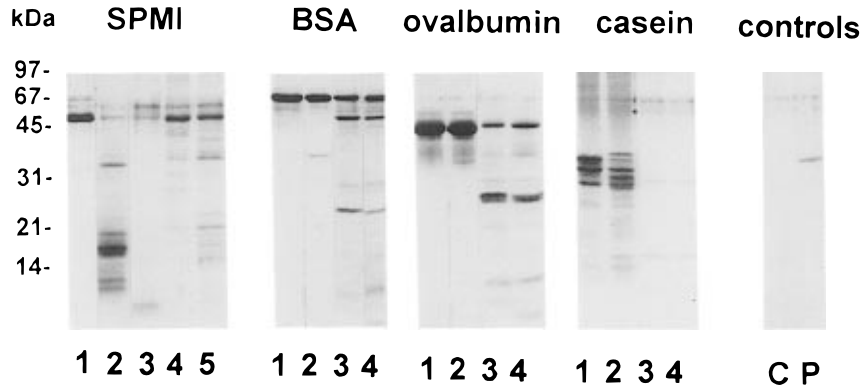

FIGURE 3: Activity of PSA and chymotrypsin on various substrates. Various protein substrates were incubated with PSA (1:25 enzyme/substrate ratio, w/w) or chymotrypsin (1:50 enzyme/substrate ratio, w/w), in the presence or absence of 1,10-phenanthroline (50 mM), for 24 h. The reaction was stopped by 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).

cysteine 216 was adjusted to take into account alkylation with iodoacetamide before purification (+57 Da), and the mass of the N-terminal residue was reduced by 17 Da to account for pyroglutamination (Lilja et al., 1989; Robert & Gagnon, 1996). 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) 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 SPMIP/SgI by PSA. Incubation of SPMIP/SgI with purified PSA caused the hydrolysis of the 52 kDa precursor (Figure 1) into multiple peptides with apparent masses below 20 kDa, while the purified 52 kDa SPMIP/SgI remained unchanged for 24 h in the absence of enzyme. When PSA was pretreated with a series of protease inhibitors, various effects were observed (Figure 1). The serine protease inhibitors PMSF and AEBSF, at a final concentration of 5 mM, prevented the hydrolysis of most of the 52 kDa SPMIP/SgI. Only low levels of proteolytic products were visible when compared with that of the untreated SPMIP/SgI. 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 SPMIP/SgI 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 SPMIP/SgI from 52 to 60 kDa. This shift in mass was accompanied by an apparent decrease in reactivity toward SPMI antiserum (Figure 1). All inhibitors were proven effective when tested on their specific target proteases, indicating that they were all active when used with PSA (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 (Figure 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 (SPMIP/SgI). Among the various protein substrates incubated with PSA, only SPMIP/SgI was significantly hydrolyzed into smaller fragments, while neither bovine serum albumin nor ovalbumin were apparently hydrolyzed by PSA (Figure 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. SPMIP/SgI was especially prone to hydrolysis by chymotrypsin. 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 SPMIP/SgI 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 (Figure 2), addition of $ZnCl_2$ (10 mM) partially reversed the inhibitory effect of 1,10-phenanthroline on SPMIP/SgI proteolysis by chymotrypsin.

Separation and Analysis of SPMIP/SgI Polypeptides Released by PSA Hydrolysis. To analyze the sites preferentially hydrolyzed by PSA, SPMIP/SgI was digested with PSA (1:50 enzyme:substrate ratio, w/w) for 24 h (Figure 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 SPMIP/SgI polypeptides since in a control run, it eluted at a

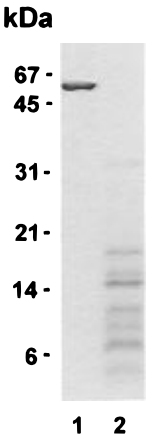

FIGURE 4: Digestion of SPMIP/SgI with PSA. The purified SPMIP/SgI dissolved in HSS (800 μg) was incubated with PSA for 24 h. An aliquot was taken, mixed with SDS sample buffer, and analyzed by SDS−PAGE as described in Materials and Methods. (1) Control SPMIP/SgI without PSA. (2) SPMIP/SgI with PSA.

concentration greater than 50% of solvent B (data not shown). The eluted fractions contained peptides with apparent masses between 6 and 20 kDa, based on SDS−PAGE analysis (Figure 5B). An aliquot of each fraction was tested for sperm motility inhibitory 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 sperm motility inhibitory activity. To identify regions of SPMIP/SgI 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 & 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 and 3−5) which also corresponded to the fractions containing the sperm motility inhibitory activity (Figure 5A,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 (Figure 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). The experimentally determined masses were then compared with the mass of peptides generated from a computer simulated digestion at the C-terminus of leucine, tyrosine, phenylalanine, and tryptophan residues of SPMIP/SgI according to the reported semenogelin sequence (Lilja et al., 1989) as described in Materials and Methods. Partial N-terminal sequences of some polypeptides were also obtained. Using this approach, the polypeptides in each fraction with masses matching those of the simulated cleavages could be assigned

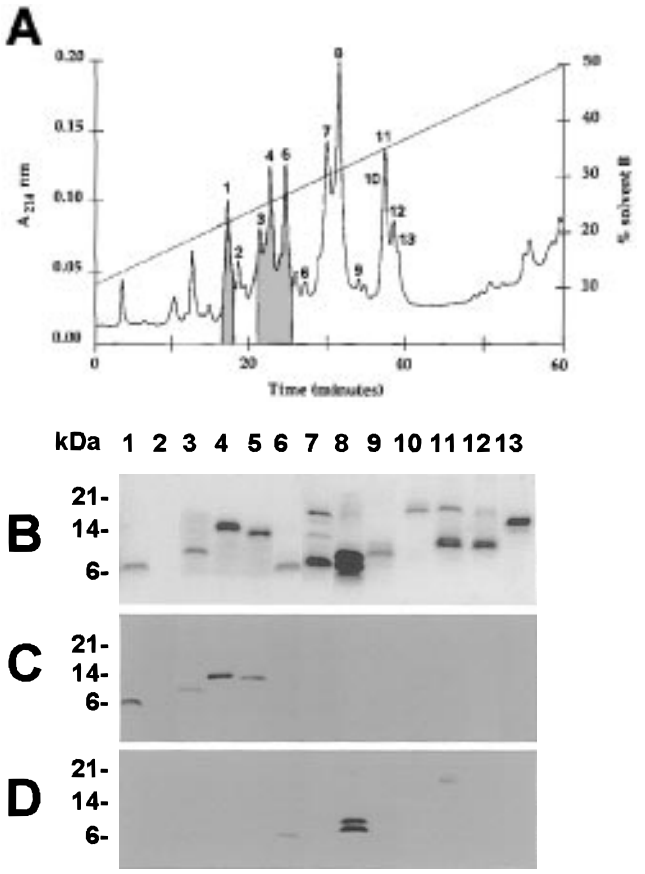

FIGURE 5: Separation and analysis of polypeptides released by hydrolysis of SPMIP/SgI by PSA. (A) The hydrolysis mixture shown in Figure 4 was made up to 0.1% TFA and injected on C_4 reverse-phase column (10 × 300 mm, 10 μm beads, 300 Å pore size) equilibrated in solvent A (0.1% TFA). Peptides were eluted with a linear gradient of 10−50% solvent B (0.1% TFA in 80% acetonitrile) over 60 min. An aliquot of each fraction was dried and analyzed for sperm motility inhibitory activity using the demembranated−reactivated sperm assay as described in Materials and Methods. Fractions that contain inhibitory activity are shaded in gray. 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 to 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 min. Number 13 corresponds to the shoulder peak at 39 min. (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).

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 1. The measured masses of polypeptides were in excellent agreement with values calculated from the cDNA sequence of semenogelin. The mass variation is within 5 mass units per 10 kDa for the majority of polypeptides. Four different polypeptides had masses that were 14−20 Da higher than the expected masses. These higher masses are likely associated with the oxidation (+16 Da) of the tryptophan residues 145 and 375 found within these polypeptides, as previously observed. In general, the measured mass for most peptides was significantly less than the apparent mass deduced by SDS−PAGE (Figure 5, Table 1).

Table 1: Identification of PSA Cleavage Sites and Resulting SPMIP/SgI Peptides

peptide	fraction number	measured mass	expected mass from cDNA[a]	polypeptide sequence	N-terminal sequence	immunoreactivity
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	10356[c]	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	9065[c]	359−439		
P14	10	14939	14922[c]	278−411		
P15	10	12640	12639	326−437		
P16	10	2770[b]	2769	1−25		
P17	11	13060	13040[c]	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[d]
P21	13	11279	11277	241−337		

[a] Mass modified to take into account N-terminal pyroglutamination and 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 of the tryptophan residues at positions 145 and 375 (+16 Da). [d] Faint reactivity.

The peptides identified in Table 1 cover the whole length of the precursor molecule and show considerable overlap. The complete sequence of SPMIP/SgI identifying the observed hydrolysis sites is shown in Figure 6. Hydrolysis of SPMIP/SgI by PSA was incomplete since not all leucine and tyrosine 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 three to six 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 seven different sites. Glutamine or glycine residues are also found three to seven residues upstream of many cleavage sites. In addition, three different hydrolysis sites were preceded by a leucine residue. 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 domains of the precursor that contain the sperm motility inhibitory activity and those recognized by the SPMI antiserum and the MHS-5 antibody. The polypeptides having inhibitory activity correspond to those recognized by the SPMI antibody, and appear to be located within a segment found between residues 85 and 136. On the other hand, the polypeptides recognized by the MHS-5 antibody originate from a different, and more centrally located, portion of the precursor between residues 198 and 223. Other polypeptides previously isolated from human seminal plasma are also shown.

DISCUSSION

PSA has been shown to degrade readily SPMIP/SgI into multiple fragments of low molecular masses (Robert & Gagnon, 1996). This degradation considerably reduces the inhibitory effect of SPMIP/SgI on sperm motility. The primary structure of PSA suggests a chymotrypsin-like specificity (Schaller et al., 1987; Lundwall & Lilja, 1987). However, previous studies on the characterization of PSA activity using synthetic or nonphysiological 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). In the present experiments, the effect of the various protease inhibitors on PSA activity toward SPMIP/SgI suggests that, although the active site of PSA is closely related to that of chymotrypsin (Vihinen, 1994), it displays significant 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 SPMIP/SgI 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/SgI serine residues may have contributed to the modifications observed. Thus the inhibitory action of NPGB on the degradation of SPMIP/SgI by PSA may result from a change in substrate conformation and/or a genuine inhibiton of PSA active site.

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 & Lutwak-Mann, 1981). Most of the results on the effect of different protease inhibitors on PSA activity measured in the present study are generally in agreement with those of previous studies on PSA activity on nonphysiological or synthetic substrates demonstrating inhibition of PSA activity by the active site reagents diisopropyl fluorophosphate (DFP) and PMSF and by zinc

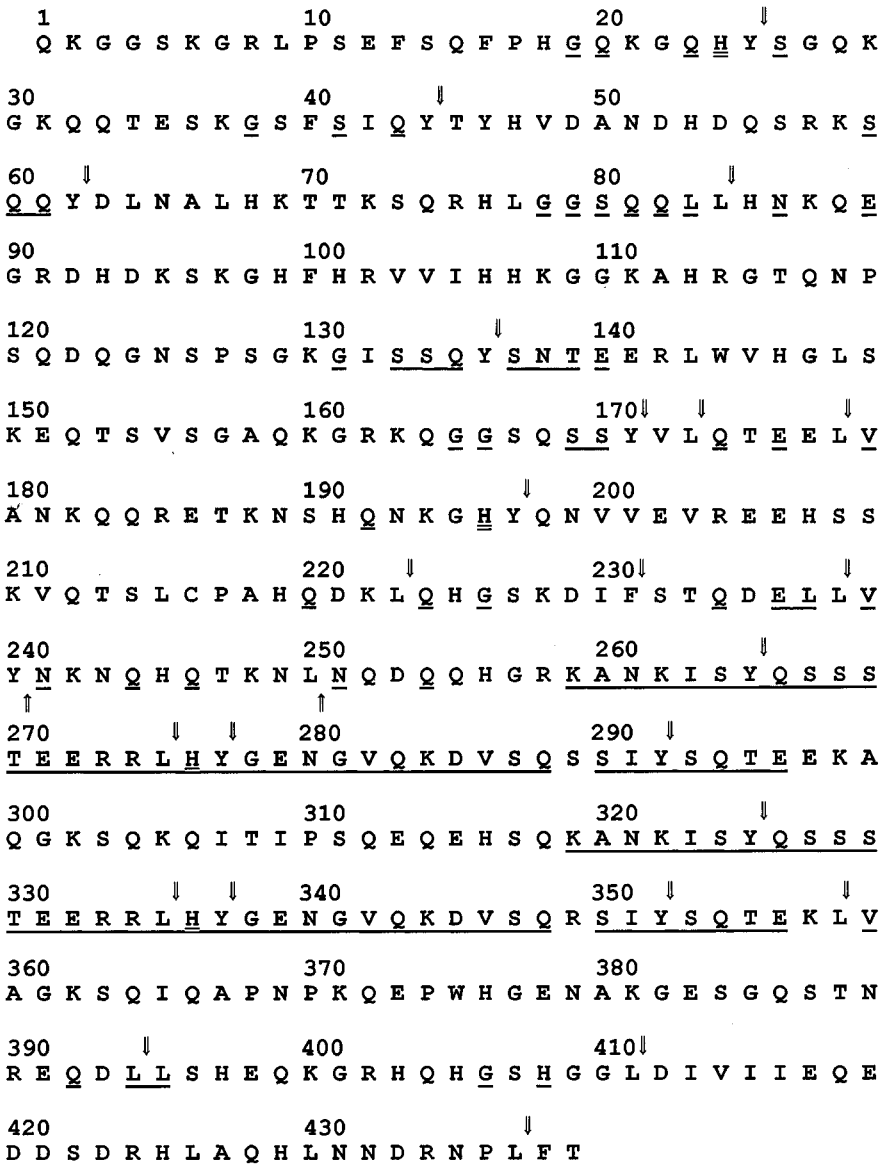

FIGURE 6: Position of PSA cleavage sites along the sequence of SPMIP/SgI. The complete SPMIP/SgI sequence (presented in the standard IUPAC, one-letter code for amino acid residues) is from Lilja et al. (1989). The PSA cleavage sites are identified by an arrow. Residues that occur at an identical position relative to the cleavage sites are underlined. Histidine residues near the cleavage sites are doubly underlined.

and mercury (Watt et al., 1986; Akiyama et al., 1987; Schaller et al., 1987).

Because a high concentration of 1,10-phenanthroline was required to inhibit the degradation of SPMIP/SgI by PSA, the possibility of a nonspecific effect of the metal chelator was entertained. However, denaturation of the enzyme or substrate by this metal chelator may be ruled out since addition of 10 mM zinc to samples containing 1,10-phenanthroline partially restored both PSA and chymotrypsin activities toward SPMIP/SgI. The chelator could, in principle, act by removing an ion that is associated with PSA and that is required for its activity. However, the observation that 1,10-phenanthroline also affected the activity of chymotrypsin, but only when SPMIP/SgI was used as the substrate, suggests that 1,10-phenanthroline chelates a metal ion associated with the substrate rather than with the enzyme. This hypothesis is reinforced by the results of previous experiments demonstrating that the major zinc ligand in seminal plasma is the predominant coagulum protein which has a mass identical to that of SPMIP/SgI (Frenette et al., 1989). Taken together, these observations suggest that SPMIP/SgI may be a zinc binding protein and that removal of the bound metal by chelators would reduce the susceptibility of SPMIP/SgI to proteolysis, possibly by inducing structural changes in the protein. The concentration of free zinc in seminal plasma is thus likely to be an important factor in the processing of SPMIP/SgI 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 SPMIP/SgI from proteolysis. The latter effect is likely related to the arrest of semen liquefaction and SPMIP/SgI degradation observed when 1,10-phenanthroline is added to freshly ejaculated semen (Lilja & Laurell, 1985; Robert & Gagnon, 1995).

PSA appears to be highly specific with respect to hydrolyzable substrates as other proteins were very poorly hydrolyzed. This may reflect a requirement for the presence of specific tertiary structures or for 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 are conserved. The activity of PSA was much lower

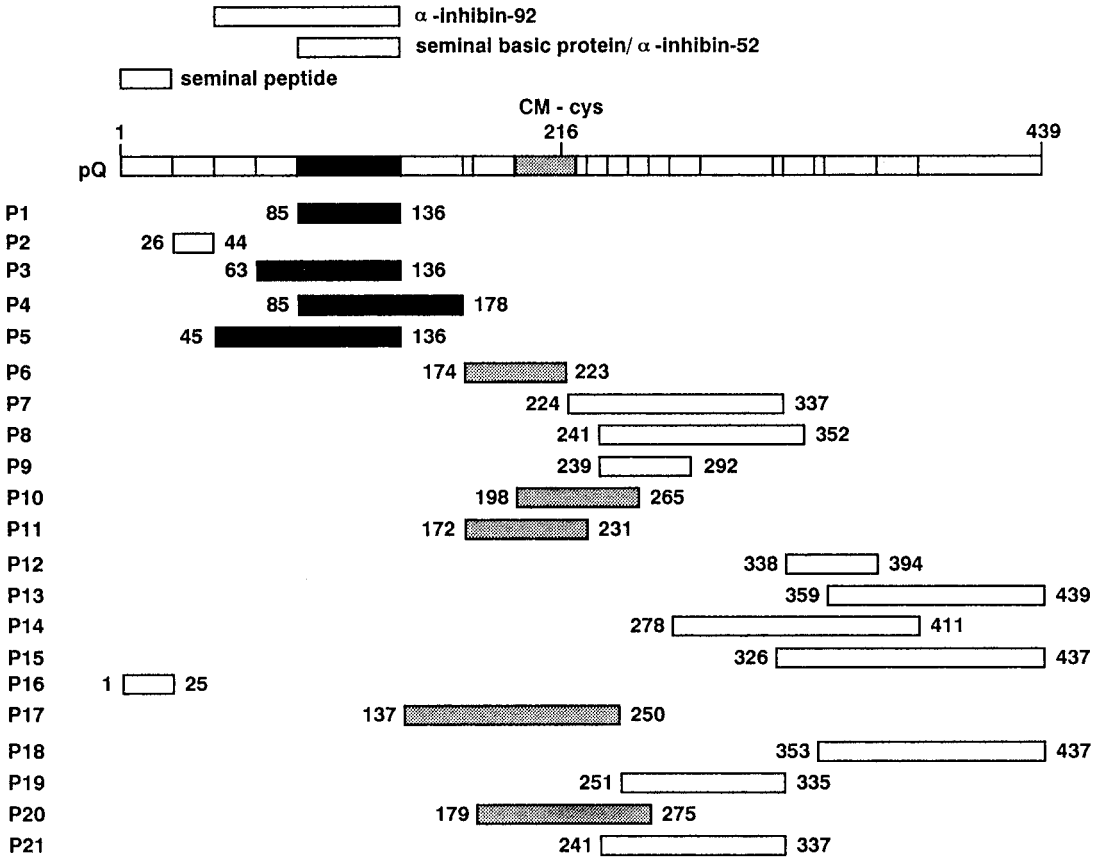

FIGURE 7: Schematic representation of SPMIP/SgI and of peptides released by PSA. The full SPMIP/SgI (residues 1−439) is shown with a carboxymethylated cysteine residue 216 (CM-cys) and a pyroglutamine residues at the N-terminus (pQ). The polypeptides identified in the present study are shown below the precursor with their N and C-terminus residue numbered. The order of presentation corresponds to that of Table 1. The polypeptides shown in black contain sperm motility inhibitory 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 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).

than that of chymotrypsin under the conditions used. These results are consistant with those of previous studies showing that PSA activity toward non physiological substrates was 200−4000 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 SPMIP/SgI hydrolysis 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 had 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 at 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 & 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 SPMIP/SgI-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.

The observation that only polypeptides derived from a common region located between amino acid 85 and 136 contained sperm motility inhibitory activity suggests that the active site is located within this specific segment of the precursor molecule. A similar conclusion can be drawn for the epitope recognized by the SPMI antiserum. The presence of this epitope in that same region (amino acid 85−136) reflects the fact that the SPMI antiserum was originally raised against a 19 kDa active SPMI form isolated from liquefied

seminal plasma (Iwamoto & Gagnon, 1988a). Two short peptides representing residues 1−25 and 26−44 of SPMIP/SgI were not detected by SDS−PAGE or immunoblots. These may be 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 SPMIP/SgI 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 and the present data suggest that SVSA is identical to SPMIP/SgI (McGee & Herr, 1987; Lilja, 1989).

It is noteworthy that some of the fragments released by PSA hydrolysis of SPMIP/SgI correspond to polypeptides that had previously been purified from seminal plasma. The 5754 Da (residues 85−136) peptide containing the sperm motility inhibitory activity 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 an N-terminal pyroglutamine residue was previously characterized (Kausler & 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 SPMIP/SgI. Finally, the possibility that SPMIP/SgI undergoes alternative processing by other seminal proteases cannot be ruled out. However, previous (Szecsi & Lilja, 1993; Robert & Gagnon, 1996) and present results coupled to the observation that many peptides which have been insolated from seminal plasma (Lilja et al., 1984; Li et al., 1985; Kausler & Spiteller, 1992) correspond to peptides observed in the present study support the notion that PSA is the main SPMIP/SgI processing enzyme in the early stage of semen liquifaction.

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