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Research Article

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A Kallikrein-like Serine Protease in Prostatic Fluid Cleaves the Predominant Seminal Vesicle Protein

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Abstract

A 33-kD glycoprotein, known as the "prostate-specific antigen," was purified to homogeneity from human seminal plasma. The prostatic protein was identified as a serine protease, and its NH_2-terminal sequence strongly suggests that it belongs to the family of glandular kallikreins. The structural protein of human seminal coagulum, the predominant protein in seminal vesicle secretion, was rapidly cleaved by the prostatic enzyme, which suggests that this seminal vesicle protein may serve as the physiological substrate for the protease. The prostatic enzyme hydrolyzed arginine- and lysine-containing substrates with a distinct preference for the former. All synthetic substrates tested were poor substrates for the enzyme. Synthetic Factor XI_a substrate (pyro-glutamyl-prolyl-arginine-*p*-nitroanilide), and the synthetic kallikrein substrate (H-D-prolyl-phenylalanyl-arginine-*p*-nitroanilide) were hydrolyzed with maximum specific activities at 23°C of 79 and 34 nmol/min per mg and K_m values of 1.0 and 0.45 mM, respectively. Synthetic substrates for plasmin, chymotrypsin, and elastase were either not hydrolyzed by the enzyme at all, or only hydrolyzed very slowly.

Introduction

A seminal coagulum forms immediately after the ejaculation of human semen. The predominant protein in seminal vesicle secretion constitutes the structural protein of the clot (1). This protein, known as the high molecular weight seminal vesicle protein (HMW-SV-protein),[1] forms disulfide-linked high molecular weight complexes but gives major protein bands of some 52, 71, and 76 kD on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of reducing agents (2, 3). The HMW-SV-protein is cleaved by proteases in the prostatic fluid to several low molecular weight proteins in concurrence with the liquefaction of the clot (1, 3). A predominant cleavage product of the HMW-SV-protein is a 5.8-kD basic protein that in its NH_2-terminal portion is similar to the histidine-rich region of bovine high molecular weight kininogen (4–6). In the rat, the NH_2-terminal portion of this basic protein is also reported to inhibit the secretion of pituitary follicle stimulating hormone (7).

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1. *Abbreviations used in this paper:* Con A, concanavalin A; DFP, diisopropylfluorophosphate; DTT, dithiothreitol; HMW-SV-protein, high molecular weight seminal vesicle protein; NPGB, 4-nitrophenyl-guanidobenzoate; *p*NA, 4-nitroanilide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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A 30-kD arginyl-cleaving serine protease is predominant in canine prostatic fluid (8, 9). Sequence homologies with glandular kallikreins strongly suggest that the canine protease is a member of the kallikrein family (10). A secretory protein from the human prostate, known as "prostate-specific antigen," has recently been shown to be a proteolytic enzyme (11). This 33-kD glycoprotein is present in human prostatic fluid in high concentration (12). Comparison of the reported NH_2-terminal sequences of the human prostatic antigen (11) and of the canine protease (10) suggests that these proteins are species analogues. If this assumption is correct, the human prostatic antigen should be a kallikrein-like protease that might cleave a kininogen-related substrate like the HMW-SV-protein. The present investigation presents results that support this hypothesis.

Methods

Reagents. Unlabeled di-isopropylfluorophosphate (DFP), 4-nitrophenyl-guanido-benzoate (NPGB)-HCl, and N^{α}-benzoyl-L-tyrosine ethyl ester were obtained from Sigma Chemical Co. (St. Louis, Mo); [^{3}H]DFP from Amersham Corp. (Amersham, United Kingdom); EN^3 HANCE from New England Nuclear (Dreieich, West Germany); pyro-glutamyl-prolyl-arginine-4-nitroanilide (*p*NA) (S 2366), H-D-prolyl-phenylalanyl-arginine-*p*NA (S 2302), H-D-valyl-leucyl-arginine-*p*NA (S 2266), H-D-phenylalanyl-pipecolyl-arginine-*p*NA (S2238), and H-D-valyl-leucyl-lysine-*p*NA (S 2251) from Kabi (Stockholm, Sweden); N^{α}-benzoyl-arginine-*p*NA from British Drug Houses (Poole, United Kingdom); and succinyl-(alanine)$_3$-*p*NA from the Protein Research Foundation (Minoh, Osaka, Japan); carboxymethyl-Sepharose, DEAE-Sepharose, and concanavalin A (Con A)-Sepharose from Pharmacia (Uppsala, Sweden); and Ultrogel Aca 54 from LKB (Bromma, Sweden).

Materials. Human semen and pure prostatic fluid were obtained as previously described (13). Pure seminal vesicle secretion was aspirated from the vesicular ducts during an operation of a male patient for carcinoma in the urinary bladder (3). Coagulated semen was collected in vessels containing 0.5 ml of 0.5 M *O*-phenanthroline that were placed in ice-cold H_2O directly after ejaculation and then frozen at −70°C until required (1). The coagulated semen was washed free of soluble components and the washed clots were dissolved, reduced, and carboxymethylated as previously described (3). Carboxymethylated clots were extensively dialyzed against 50 mM Tris-HCl with 0.5 M NaCl, pH 8.0.

Electrophoretic techniques. Agarose gel electrophoresis was performed according to standard procedures (14). SDS-PAGE in a 9–17% polyacrylamide gel gradient was performed according to Blobel and Dobberstein (15). Gels with radioactive proteins were dried after protein fixation and treatment with EN^3 HANCE according to the manufacturer's instructions, and then exposed for 30 d to x-ray films at −70°C before development. Throughout the purification procedures the individual fractions were analyzed with electroimmunoassay using a polyclonal rabbit antiserum raised against the purified prostatic 33-kD glycoprotein. The electroimmunoassay was run at 60 V for 16 h in gels that contained 1% of antiserum.

Protein purification. The 33-kD prostatic glycoprotein was purified from 50 ml of pooled seminal plasma. The seminal plasma pool was diluted to 150 ml and made up to 10 mM with benzamidine. Concentrations on Diaflo membranes (Amicon Corp., Danvers, MA) (cut off range = 5 kD) and all chromatographic steps were carried out at 4°C.

All buffers used were made up to 10 mM with benzamidine. The seminal plasma pool was applied to a carboxymethyl-Sepharose column (40 × 2.5 cm) equilibrated with 50 mM Tris-HCl, pH 7.5. The prostatic glycoprotein passed through the column with the equilibrating buffer. Fractions containing the protein were pooled and concentrated to ~100 ml. This pool was applied to a DEAE-Sepharose column (30 × 2.5 cm) equilibrated with 30 mM sodium phosphate, pH 6.4. The prostatic glycoprotein passed through the column with the equilibrating buffer and the fractions were pooled and concentrated. The pool was subsequently applied to a Con A-Sepharose column (16 × 2.5 cm) equilibrated with 50 mM Tris also containing 0.5 M NaCl, pH 7.5. The prostatic glycoprotein was eluted from the column with 0.2 M α-methyl-mannoside added to the equilibrating buffer and the fractions were pooled and concentrated (Fig. 1 *A*). The protein pool was applied to an Ultrogel Aca 54 column (88 × 1.6 cm) equilibrated with 50 mM Tris and 0.5 M NaCl, pH 8.0 (Fig. 1 *B*). Fractions reacting with the antibody were pooled and the resulting material found to contain a single band of ~33 kD as judged by SDS-PAGE of reduced samples (Fig. 2 *A*). The recovery after each purification step is given in Table I.

Enzyme assays. Hydrolysis of the tripeptide *p*Na-substrates and N^{α}-benzoyl-arginine-*p*NA was measured with a UV 260 recording spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto, Japan) at 405 nm. Hydrolysis of N^{α}-benzoyl-L-tyrosine ethyl ester was measured at 253 nm. All incubations were performed at 23°±2°C in 50 mM Tris, pH 7.8. The reaction was initiated by the addition of the purified protein to 1 ml of substrate solution, while the absorbance was monitored for at least 20 min against a blank containing the substrate solution.

Labeling with [^{3}H]DFP. Samples of purified protein (each containing ~4 μg), pure prostatic fluid (4 μl), and seminal plasma (4 μl) were incubated with 3 μl of [^{3}H]DFP (5 mCi/ml) in 15 μl of 50 mM Tris-HCl, pH 7.8, for 10–30 min at room temperature. The samples were subsequently analyzed using SDS-PAGE after reduction and carboxymethylation.

Titration of enzymatic activity. Active site titration with NPGB was performed according to Chase and Shaw (17). The molar concentration of the purified protein was calculated from amino acid analysis assuming a mass of 33 kD and a carbohydrate content of 10% (12).

Amino acid analysis and sequence determination. The purified protein was reduced for 1 h with 25 mM dithiothreitol (DTT) in 0.5 M Tris-HCl with 6 M guanidine-HCl, pH 8.0, and then carboxymethylated for 2 h with 50 mM iodoacetic acid in 0.5 M Tris, pH 8.0. Carboxymethylated protein was hydrolyzed in 6 M HCl in evacuated sealed tubes at 110°C for 24 h. The hydrolysates were analyzed in an amino acid analyzer (model 6300; Beckman Instruments Inc., Fullerton, CA). The NH_2-terminal sequence of ~40 μg of carboxymethylated protein was analyzed using a 470A gas phase sequencer (Applied Biosystems, Inc., Foster City, CA) with a standard program (18). All phenylthiohydantoin-amino acids were identified with HPLC on a Novapak C_{18} column (Waters Instruments, Inc., Rochester, MN). The column was equilibrated in 29 mM sodium acetate, pH 5.05, with 17% acetonitrile, and eluted with a linear gradient against 60% isopropanol.

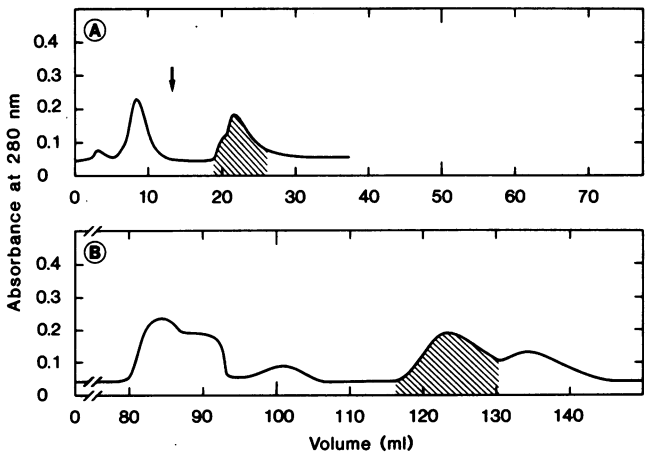

Figure 1. (*A*) Affinity chromatography on Con A-Sepharose at a flow rate of 25 ml/h of pooled fractions from DEAE-Sepharose containing the prostatic 33-kD glycoprotein. Elution with 0.2 M α-methyl-D-mannoside was started at ↓. The hatched peak contained the 33-kD glycoprotein. (*B*) Gel filtration on Ultrogel AcA 54 at a flow rate of 3 ml/h of the pooled fractions from *A*. The fractions that were pooled, concentrated, and analyzed by SDS-PAGE in Fig. 2 *A* have been indicated by hatching.

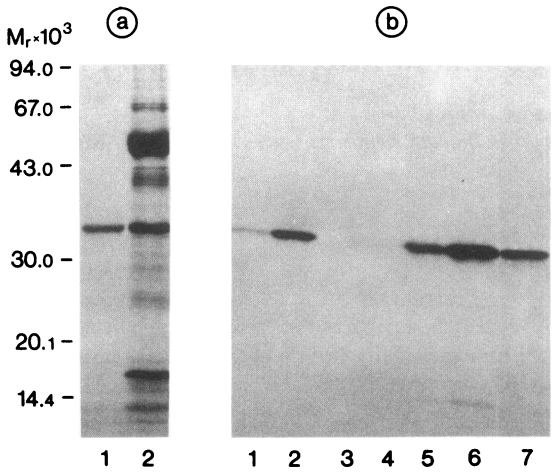

Figure 2. (*A*) SDS-PAGE in a 9–17% polyacrylamide gel gradient. All samples were reduced (10 mM DTT) and carboxymethylated before electrophoresis. The proteins were stained with Coomassie Blue R 250 after completed electrophoresis. Lane *1*, Purified prostatic protein (~15 μg); lane *2*, prostatic fluid (4 μl). (*B*) Fluorographic enhancement after SDS-PAGE. Lane *1*, purified protein (5 μg) incubated with [^{3}H]DFP for 10 min; lane *2*, purified protein (5 μg) incubated with [^{3}H]DFP for 30 min; lane *3*, purified protein (5 μg) treated with unlabeled DFP (10 mM) before incubation with [^{3}H]DFP for 30 min; lane *4*, purified protein (5 μg) reduced and carboxymethylated before incubation with [^{3}H]DFP for 30 min; lane *5*, prostatic fluid (4 μl) incubated with [^{3}H]DFP for 10 min; lane *6*, prostatic fluid (4 μl) incubated with [^{3}H]DFP for 30 min; lane *7*, seminal plasma (4 μl) incubated with [^{3}H]DFP for 30 min.

Results

Identification of the purified protein. Analysis of the 15 NH_2-terminal amino acid residues of the purified protein gave the

Table I. Purification of Prostatic 33-kD Glycoprotein

Steps	Total volume	Total protein	Total amount of 33-kD glycoprotein	Recovery
	ml	*mg*	*mg*	*%*
Seminal plasma	50	2,300	63	100
CM-Sepharose	100	600	52	82
DEAE-Sepharose	7.0	130	26	41
Con A-Sepharose	4.0	32	18	28
Ultrogel AcA 54	4.2	13	12	19

CM, carboxymethyl.
The amount of prostatic 33-kD glycoprotein was determined by electroimmunoassay. The assay was calibrated by the use of amino acid analysis of the pooled fraction from AcA 54. Total protein was determined according to Lowry (16) with the use of albumin as standard.

following sequence: Ile-Val-Gly-Gly-Trp-Glu-Cys-Glu-Lys-His-Ser-Gln-Pro-Trp-Gln. The yield of the first amino acid (0.9 nmol of Ile) corresponded to ~75% of the calculated theoretic yield.

Enzymatic activity of the purified protein. The purified protein did not hydrolyze N^{α}-benzoyl-tyrosine ethyl ester or succinyl-(alanine)$_3$*p*NA. Kinetic data for various synthetic substrates are given in Table II. DFP added to the purified protein in 200-fold molar excess abolished its hydrolysis of H-D-prolyl-phenylalanyl-arginine-*p*NA and pyro-glutamyl-prolyl-arginine-*p*NA. Active site titration of the purified protein with NPGB demonstrated that 66% of the protein had enzymatic activity.

Incorporation of [^{3}H]DFP in the purified protein. Fluorographic enhancement after SDS-PAGE of the purified protein showed that it incorporated [^{3}H]DFP in a time-dependent manner (Fig. 2 *B*). The incorporation of [^{3}H]DFP was blocked if the purified protein had been treated with unlabeled DFP (10 mM), or if it had been carboxymethylated before it was incubated with the radioactive compound. One single band, with the same mass as the purified protein, incorporated [^{3}H]DFP both in pure prostatic secretion, and in seminal plasma as judged by SDS-PAGE (Fig. 2 *B*).

Cleavage of HMW-SV-protein by the purified protein. Native HMW-SV-protein (in seminal vesicle secretion) and carboxymethylated HMW-SV-protein (from seminal clots) were analyzed by SDS-PAGE (Fig. 3). The intact HMW-SV-protein gave three major bands of 52, 71, and 76 kD, as marked with arrows in the figure. The series of minor bands with molecular masses below 50 kD (lanes 1 and 6 in Fig. 3) are intermediate cleavage products of the HMW-SV-protein as previously demonstrated in seminal clots by immunoblotting after SDS-PAGE (1, 3). The seminal vesicle secretion and seminal clots were incubated at 37°C with the purified 33-kD prostatic protein (3 μg). The purified protein rapidly cleaved native or carboxymethylated HMW-SV-protein as shown by the fast disappearance of the 52- and 76-kD bands. The 71-kD band of the intact protein was somewhat more resistant to cleavage (lanes 2–4 and 7 in Fig. 3). Below the weakly stained 71-kD band of HMW-SV-protein in lane 7 a faint 67-kD band of albumin is seen. Proteins with molecular masses below 20 kD appeared in conjunction with the disappearance of intact HMW-SV-protein. On agarose gel electrophoresis the cleavage of HMW-SV-protein by the purified prostatic protein yielded a series of basic proteins that were indistinguishable from the basic proteins of liquefied semen (Fig. 4). The basic low molecular mass degradation products are HMW-SV-related proteins, as previously shown by immunoblotting (1, 3). In addition, acidic cleavage products also appeared when seminal clots (Fig. 4) or seminal vesicle secretion (not shown) were incubated with the purified prostatic protein. The 5.8-kD predominant basic protein in seminal plasma was one of the end products formed after 60 min (Figs. 3 and 4). However, purified 5.8-kD basic protein was not cleaved further by the purified protein (not shown).

Table II. Hydrolysis of Synthetic Substrates by the Purified Protein

Substrate	V_{max}	K_m	K_{cat}	K_{cat}/K_m
	nmol/min/mg	*mM*	s^{-1}	$s^{-1}M^{-1}$
Pyro-glutamyl-prolyl-arginine-*p*NA	79	1.0	0.064	64
H-D-prolyl-phenylalanyl-arginine-*p*NA	34	0.45	0.028	62
H-D-phenylalanyl-pipecolyl-arginine-*p*NA	36	0.59	0.029	49
H-D-valyl-leucyl-arginine-*p*NA	34	4.0	0.028	7
H-D-valyl-leucyl-lysine-*p*NA	2.9	—	—	—
N^{α}-benzoyl-arginine-*p*NA	7.5	—	—	—

All assays were performed at 23°±2°C and pH 7.8 as described in Methods. Michaelis-Menten constants were obtained from Lineweaver-Burk plots of rates of hydrolysis. Hydrolysis of H-D-valyl-leucyl-lysine-*p*NA and N^{α}-benzoyl-arginine-*p*NA were measured at a substrate concentration of 1 mM.

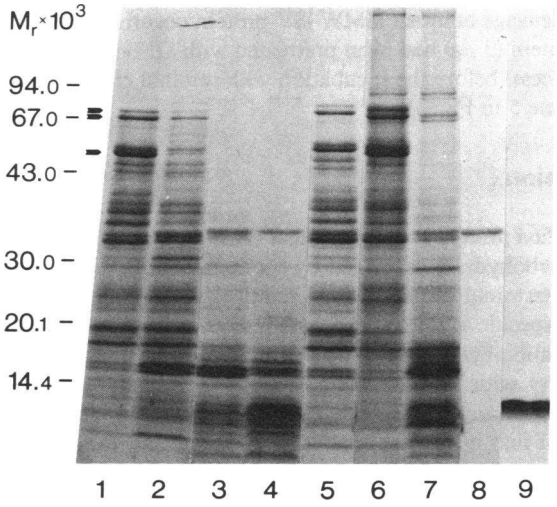

Figure 3. SDS-PAGE in a 9–17% polyacrylamide gel gradient. All samples were reduced (10 mM DTT) and carboxymethylated before electrophoresis. The proteins were stained with Coomassie Blue R 250 after completed electrophoresis. Lane *1*, dissolved, reduced, and carboxymethylated seminal clot (10 μl); lane *2*, purified prostatic protein (3 μg) incubated with the carboxymethylated clot (10 μl) at 37°C, for 2 min; lane *3*, as in *2* but the sample was incubated for 15 min; lane *4*, as in *2* but the sample was incubated for 60 min; lane *5*, purified prostatic protein (3 μg) treated with DFP (10 mM) before it was mixed with the carboxymethylated clot (10 μl) and incubated at 37°C for 30 min; lane *6*, pure seminal vesicle secretion (2 μl); lane *7*, purified prostatic protein (3 μg) incubated with the seminal vesicle secretion (2 μl) at 37°C, for 5 min; lane *8*, purified prostatic protein (5 μg); lane *9*, purified 5.8-kD predominant basic protein.

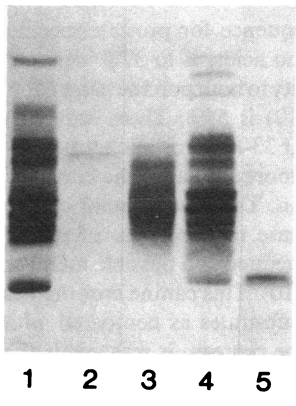

Figure 4. Agarose gel electrophoresis at pH 8.6. The proteins were fixed and stained with Coomassie Blue R 250 after completed electrophoresis. Anode at top. Lane *1*, liquefied semen (4 μl); lane *2*, purified prostatic protein (3 μg); lane *3*, dissolved, reduced, and carboxymethylated seminal clot (10 μl); lane *4*, purified prostatic protein (3 μg) incubated with the carboxymethylated clot (10 μl) at 37°C, for 60 min; lane *5*, purified 5.8-kD predominant basic protein.

No cleavage of intact HMW-SV-protein occurred if the purified protein (3 μg) had been pretreated with DFP (in 200-fold molar excess) before the incubation with seminal clots was initiated (lane 5 in Fig. 3).

Discussion

The purified protein had a molecular mass of ~33 kD and contained carbohydrate as judged by the high affinity for Con A. Its NH_2-terminal sequence was identical to that reported for prostate-specific antigen (11). These results, in conjunction with the prostatic origin of our purified protein, provide evidence of its identity with the prostate-specific antigen. Ban et al. (11) reported that prostate-specific antigen possessed proteolytic activity, but that it was not a serine protease, although the NH_2-terminal sequence was very similar to serine proteases, particularly to rat submaxillary tonin (19). Our results clearly demonstrate that the purified protein was a serine protease. DFP destroyed the functional activity of the purified protein. It incorporated [^{3}H]DFP in a specific and time-dependent manner. The result from titration of enzymatic activity excludes the possibility that the demonstrated [^{3}H]DFP incorporation may have been due to a minor contaminant present in the purified material. Furthermore, the sequence (-Val-Leu-Thr-Ala-Ala-His-Cys-) reported for prostate-specific antigen by Ban et al. (11) suggests that His 41 is a member of the catalytic triad of serine proteases (20). The purified protein had no chymotryptic (tyrosine-cleaving) or elastolytic (alanine-cleaving) activity. It possessed a substrate specificity restricted to arginine- and lysine-containing substrates, but with a distinct preference for the former. Synthetic plasma kallikrein-substrate (Pro-Phe-Arg-*p*NA) and Factor XI_a-substrate (Glu-Pro-Arg-*p*NA) were the most efficient, but all synthetic substrates tested were poor substrates for the purified protein. However, the prostatic enzyme very rapidly cleaved intact HMW-SV-protein, as judged by the fast disappearance of the 52-, 71-, and 76-kD bands on SDS-PAGE. The presented results do not permit description of one separate band being transformed to another, because of the complexity of the HMW-SV-protein cleavage. The enzyme-to-substrate concentration ratio used for the electrophoretic mapping of the HMW-SV-protein cleavage by the purified enzyme was comparable to their physiological ratio in ejaculated semen. The alterations of the protein pattern resulting from cleavage of HMW-SV-protein by the prostatic enzyme were very similar to the normal proteolysis of HMW-SV-protein in semen (3). It may, therefore, be concluded that the HMW-SV-protein serves as a, or perhaps the, physiological substrate for the purified prostatic protein when prostatic fluid is mixed with the seminal vesicle secretion.

The reported NH_2-terminal sequence for prostate-specific antigen (11), consisting of 68 amino acids, is to 37% identical to porcine trypsin (21), but the identity to both porcine pancreatic kallikrein (22) and to rat tonin (19) is 54%. These sequence homologies strongly suggest that our 33-kD prostatic serine protease (prostate-specific antigen), is more similar to the family of glandular kallikreins than to trypsin. The predominant canine prostatic enzyme is a serine protease that, because of partial sequence analysis, has also been suggested as a possible member of the glandular kallikrein family (10). This canine enzyme has similar specificities for synthetic substrates as compared with our human protease (8). The canine protease is reasonably the species analogue to our purified protein, as their masses and NH_2-terminal sequences are very similar (10, 11).

The NH_2-terminal portion of the predominant 5.8-kD cleavage product of the HMW-SV-protein has sequence homologies with two portions of the histidine-rich region of bovine high molecular weight kininogen (4). The data presented here indicate that a system consisting of a kallikrein-like enzyme and a kininogen-like substrate is activated at the ejaculation of human semen. It is of pertinent interest to study the sperm interactions of the liberated cleavage products of the HMW-SV-protein.

Acknowledgments

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