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Human prostate-specific antigen: Structural and functional similarity with serine proteases

(primary structure/sequence homology/seminal protease/chymotrypsin-like and trypsin-like activity)

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ABSTRACT **The complete amino acid sequence of the prostate-specific antigen (PA) from human seminal plasma has been determined from analyses of the peptides generated by cyanogen bromide, hydroxylamine, endoproteinases Arg-C and Lys-C. The single polypeptide chain of PA contains 240-amino acid residues and has a calculated M_r of 26,496. An N-linked carbohydrate side chain is predicted at asparagine-45, and O-linked carbohydrate side chains are possibly attached to serine-69, threonine-70, and serine-71. The primary structure of PA shows a high degree of sequence homology with other serine proteases of the kallikrein family. The active site residues of histidine, aspartic acid, and serine comprising the charge-relay system of typical serine proteases were found in similar positions in PA (histidine-41, aspartic acid-96, and serine-192). At pH 7.8, PA hydrolyzed insulin A and B chains, recombinant interleukin 2, and—to a lesser extent—gelatin, myoglobin, ovalbumin, and fibrinogen. The cleavage sites of these proteins by PA were chemically analyzed as the α-carboxyl side of some hydrophobic residues, tyrosine, leucine, valine, and phenylalanine, and of basic residues histidine, lysine, and arginine. The chymotrypsin-like activity of PA exhibited with the chromogenic substrate *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide yielded a specific activity of 9.21 μM per min per mg of PA and K_m and k_{cat} values of 15.3 mM and 0.075 s^{-1}, respectively. "Trypsin-like" activity of PA was also detected with Nα-benzoyl-DL-arginine *p*-nitroanilide and gave a specific activity of 1.98 μM per min per mg of PA. Protease inhibitors such as phenylmethylsulfonyl fluoride, diisopropyl fluorophosphate, L-1-tosylamido-2-phenylethyl chloromethyl ketone, aprotinin, leupeptin, soybean trypsin inhibitor as well as Zn^{2+} and spermidine were effective inhibitors of PA enzymatic activity.**

Human prostate-specific antigen (PA) is a glycoprotein with a M_r of 33,000–34,000 containing approximately 7% (wt/wt) carbohydrate (1, 2). PA is detected only in the epithelial cells of the prostatic ductal element by a specific antibody to PA (1, 3). Seminal plasma also contains PA identical to that of the prostate gland (3). PA is synthesized in the epithelial cells of the prostate gland and secreted into the seminal fluid (3). Recently, PA has been demonstrated to be clinically important for the detection and monitoring of prostate cancer (4, 5). The PA molecule was independently identified as p30 (6) and was tested as a marker for postcoital detection in rape investigations (7). Although the clinical utility of PA has been shown, its biological function and chemical structure are not well characterized (8). We report here the complete amino acid sequence of PA and describe the characteristics of its enzymatic properties.

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MATERIALS AND METHODS

PA was purified from human seminal plasma as described (1). PA was finally purified on a large-pore Vydac (Hesperia, CA) C_4 column and eluted either with a linear gradient between 70% (vol/vol) buffer A (buffer A = 0.1% trifluoroacetic acid in H_2O) and 37% (vol/vol) buffer B (buffer B = 0.1% trifluoroacetic acid in acetonitrile) in 280 min or 10% (vol/vol) buffer B and 80% (vol/vol) buffer B in 60 min.

The enzymatic activity of PA was assessed on commercially purified proteins including insulin A, insulin B, gelatin, myoglobin, ovalbumin, fibrinogen (Sigma), and recombinant interleukin 2 (Ala-IL2, Cetus). The substrates (1 mg/ml) were dissolved in either 0.1 M ammonium bicarbonate or 50 mM Tris·HCl, pH 7.8, containing PA at 0.1 mg/ml. In some cases, a mass ratio (enzyme:substrate) of 1:20 was used. After an 18-hr digestion at 37°C, the peptides were separated by HPLC using a 60-min linear gradient of 0–80% (vol/vol) buffer B. To determine the peptide bond specificity of PA, hydrazinolysis was performed on each of the PA/substrate digestion mixtures, substrate alone, and intact PA as described (9). The free carboxyl-terminal amino acids were lyophilized and analyzed on a Beckman 121MB amino acid analyzer.

The kinetics of PA hydrolytic activity on synthetic substrates were studied by monitoring the absorbance change at room temperature using a Hewlett–Packard 8450A spectrophotometer. The following substrates were used: Nα-benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt), Nα-succinyl-phenylalanine nitroanilide (*N*-Suc-Phe-NA), Nα-benzoyl-arginine-β-naphthylamide (Bz-Arg-NHNpt), Nα-benzoyl-DL-arginine-*p*-nitroanilide (Bz-Arg-*p*-NA), Nα-tosyl-L-arginine methyl ester (Tos-Arg-OMe) (Sigma), and Nα-succinyl-L-alanyl-L-alanyl-L-prolyl-Lphenylalanine-*p*-nitroanilide (*N*-Suc-Ala-Ala-Pro-Phe-*p*-NA) (Vega Biochemical, Tucson, AZ). The K_m and the k_{cat} of PA on *N*-Suc-Ala-Ala-Pro-Phe-*p*-NA were calculated from Lineweaver–Burke plots. Inhibitors at 2 mM in 0.1 M $CaCl_2$/0.1 M Tris·HCl (pH 8.0) were used to affect the PA activity on these substrates. The following inhibitors were tested: phenylmethylsulfonyl fluoride ($PhMeSO_2F$), diisopropylfluorophosphate (DFP), L-1-tosylamido-2-phenylethyl chloromethyl ketone (Tos-$PheCH_2Cl$), aprotinin, soybean trypsin inhibitor, zinc acetate, spermidine (Sigma), and leupeptin (Boehringer Mannheim).

The primary structure of PA was established by automated sequence analyses of intact polypeptide and by characterization of CNBr, hydroxylamine, endoproteinase Arg-C, and endoproteinase Lys-C peptides. CNBr reaction was conduct-

Abbreviations: *N*-Suc-Ala-Ala-Pro-Phe-*p*-NA, Nα-succinyl-L-alanyl-L-analyl-L-prolyl-L-phenylalanine-*p*-nitroanilide; Bz-Arg-*p*-NA, Nα-benzoyl-DL-arginine-*p*-nitroanilide; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; DFP, diisopropylfluorophosphate; Tos-$PheCH_2Cl$, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Bz-Tyr-OEt, Nα-benzoyl-L-tyrosine ethyl ester; *N*-Suc-Phe-NA, Nα-succinyl-phenylalanine nitroanilide; Bz-Arg-NHNpt, Nα-benzoyl-arginine-β-naphthylamide; Tos-Arg-OMe, Nα-tosyl-L-arginine methyl ester; PA, prostate-specific antigen.

ed by dissolving the protein (5 mg/ml) in 70% (vol/vol) formic acid with the addition of solid CNBr (2-fold excess by weight) for 24 hr at room temperature. Hydroxylamine cleavage was carried out according to Bornstein and Balian (10). Digestion of PA (5 mg/ml) with endoproteinase Arg-C and endoproteinase Lys-C (Boehringer Mannheim) was performed in 0.1 M ammonium bicarbonate containing 0.2 mg of enzyme per ml at 37°C for 18 hr. All peptides were purified by HPLC on a Vydac C_4 column using the F_3CCOOH/CH_3CN buffer system with a linear gradient from 100% buffer A to 80% (vol/vol) buffer B in 60 min. Amino acid analyses were performed using a Beckman 121MB amino acid analyzer after 24, 48, and 72 hr of acid hydrolysis (5.7 M HCl, 0.1% phenol). Automated sequence analyses were conducted on a Beckman 890C sequencer and Applied Biosystems (Foster City, CA) 470A gas-phase sequencer as reported (11). Comparison of the primary structure of PA with the kallikrein family of serine protease was carried out according to Doolittle (12) and Dayhoff (13).

RESULTS

Amino Acid Composition. The amino acid composition of PA is tabulated in Table 1. Our results agree well with a previous report and with the theoretical composition derived from the sequence reported here.

Amino Acid Sequence. The complete amino acid sequence of PA is given in Fig. 1. The sequence was primarily constructed through analysis of the reduced and alkylated chain, of fragments derived from partial endoproteolytic cleavages, CNBr fragmentation, endoproteinase Arg-C, and Lys-C cleavages. Amino-terminal sequence analysis with *o*-phthalaldehyde blocking at proline residues (11) yielded the sequence of R1–R87. The chemical cleavage of methionine residues and enzymatic digestion with endoproteinase Lys-C gave the sequences of R1–R170, R174–R198, and R225–R240. The sequence between residues 199 and 224 was furnished by an additional chemical cleavage with hydroxylamine at the unique asparagine-glycine sequence (R199–R200). The sequence of the tripeptide (R171–R173)

Table 1. Amino acid composition of human prostate antigen

Amino acid	Residues per 240 residues total		
	Ref. 2*	This report†	From sequence
Cys	7.8	9.9‡	10
Asx	16.9	16.9	16
Thr	12.8	13.4	13
Ser	21.8	18.8	19
Glx	25.1	22.8	23
Pro	16.6	16.0	16
Gly	25.7	20.0	20
Ala	14.4	11.9	12
Val	20.4	21.2	22
Met	3.7	4.2	5
Ile	8.1	8.7	8
Leu	24.4	25.6	26
Tyr	4.1	4.7	5
Phe	6.7	6.4	5
Lys	10.7	12.0	12
His	11.2	11.0	11
Trp	2.4	5.5‡	7
Arg	9.5	9.5	10
Total	242.3	238.5	240

*Recalculated from the original data on the basis of 240 residues.
†The data represent averages of duplicate analyses of four different PA preparations after 24, 48, and 72 hr of acid hydrolysis.
‡Cysteine values were obtained as cysteic acid after performic acid oxidation. Tryptophan values were obtained after a 24-hr hydrolysis with 5.7 M HCl/10% (vol/vol) mercaptoacetic acid.

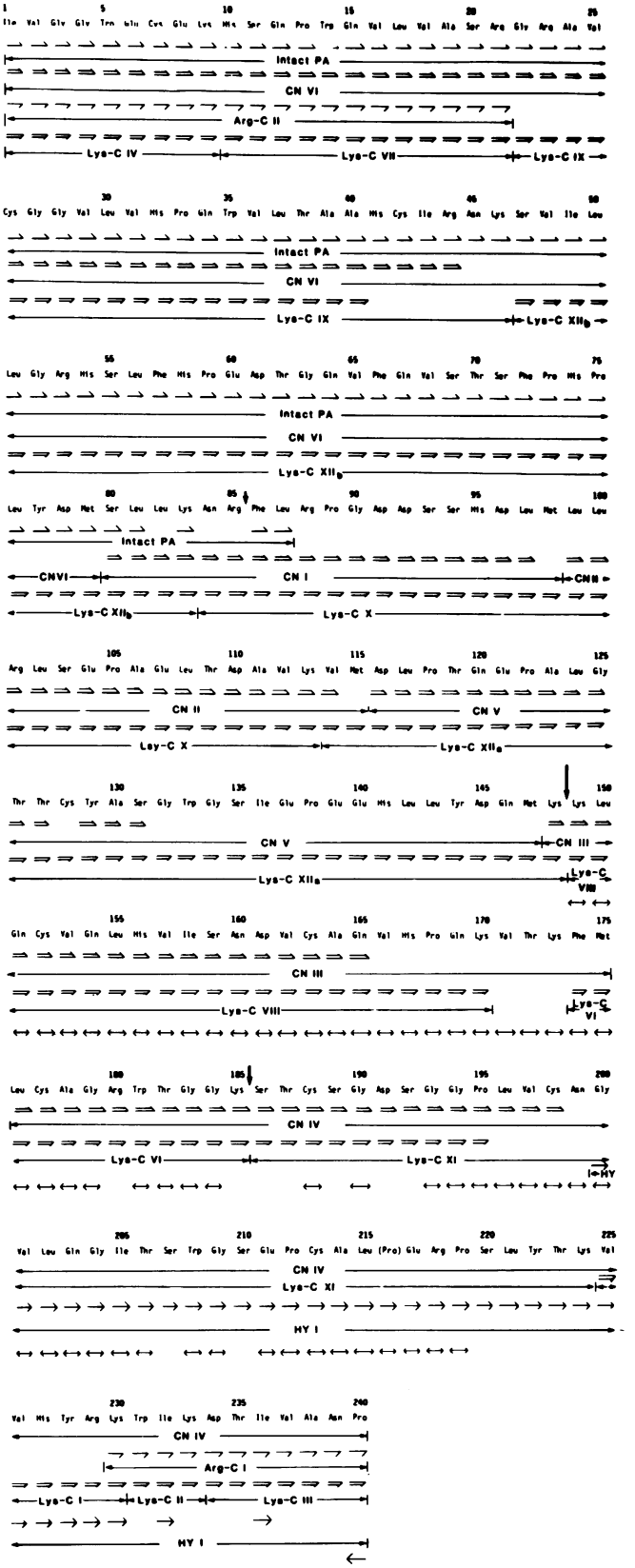

FIG. 1. Schematic presentation of the evidence for sequence assembly of human PA. Peptides were generated by cleavage with CNBr, endoproteinases Arg-C or Lys-C, or hydroxylamine (HY). Arrows above the designated peptides indicate sequenced residues. →, intact PA; ⇒, CNBr peptides; →, Arg-C peptides; ⇉, Lys-C peptides; →, HY peptide; ↔, 2 major internal proteolytic cleavages; and ←, carboxyl-terminal analysis by hydrazinolysis. The vertical arrows indicate the three proteolytic cleavage sites contributing to 10–20% of the total naturally occurring forms.

was determined from the sequence analysis of the internal fragment, during the sequencing of the intact molecule, as a result of the major but partial endoproteolytic cleavage between residue 148 and 149. The peptide Arg-CI overlapped the two carboxyl-terminal Lys-C peptides (Fig. 1). This sequence, together with the carboxyl-terminal proline residue obtained by hydrazinolysis of the intact molecule, showed that the primary structure of PA ends at residue 240.

Endoproteolytic Cleavages. Automated Edman degradation of reduced and alkylated HPLC-purified PA always gave the amino-terminal tetrapeptide sequence of Ile-Val-Gly-Gly.

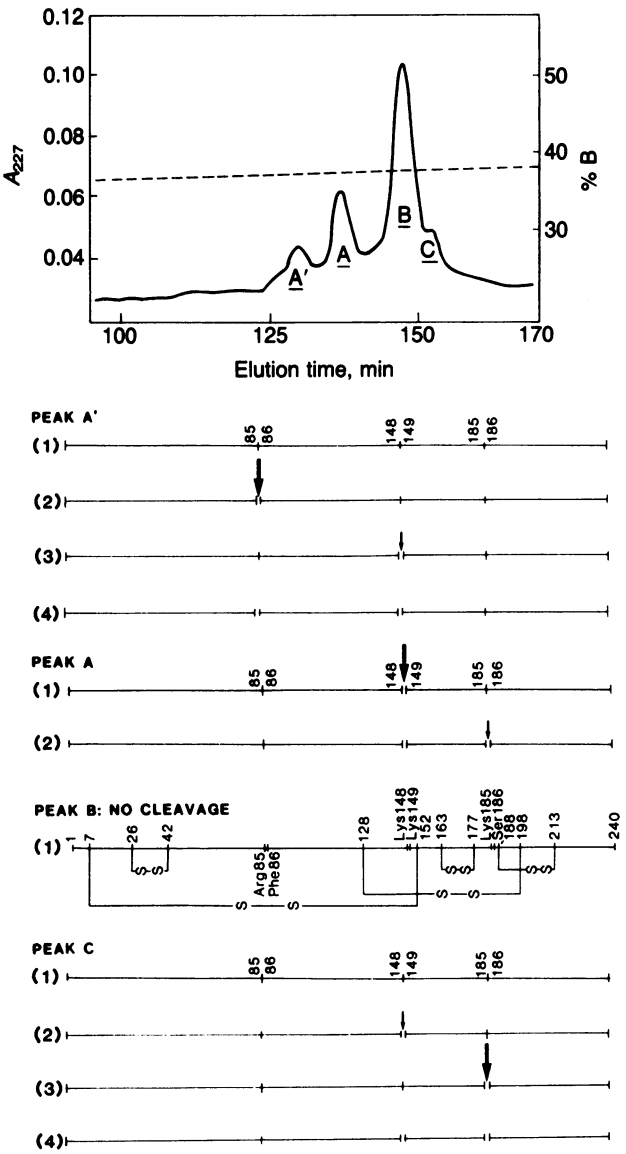

FIG. 2. Separation of naturally occurring forms of human PA as a result of endoproteolytic cleavages. A very shallow organic gradient of 0.025% per min [30–37% (vol/vol) buffer B in 280 min] was used to elute the HPLC-purified PA sample on a Vydac C_4 column. Four pools designated A', A, B, and C were obtained, lyophilized, and subjected separately to Edman degradation. The different PA forms were derived from the stepwise yield of phenylthiohydantoin amino acid derivatives in each pool. The bold arrow denotes a higher yield of cleavage, and the small arrow denotes a lower yield in each pool. Thus, peptide 1 in peaks A', B, and C; peptide 2 in peak A'; peptide 3 in peak A' is peptide 1 in peak A and peptide 2 in peak C; peptide 4 in peak A'; peptide 2 in peak A is peptide 4 in peak C; and peptide 3 in peak C represent six out of a possible total of eight forms of PA resulting from three endoproteolytic sites in the PA molecule held together by internal disulfide linkages.

However, two minor sequences of Lys-Leu-Gln-Cys and Ser-Thr-Cys-Ser were also found. These minor sequences are consistent with peptide bond cleavages in PA at lysine-148 and lysine-185, respectively. Some of the molecules contained a third cleavage following arginine-85 (Fig. 1). These three cleavage sites were present in 10–20% of the total naturally occurring forms; the remaining 80% represented the single chain of PA without any internal clips. There were differences in the relative amounts of PA containing cleavages at lysine-148, lysine-185, and arginine-85. In general, the pattern of cleavage was lysine-148 > lysine-185 > arginine-85. Therefore, PA is a mixture of molecules containing one, two, three, or four chains attached covalently by disulfide bridges that arise from one, two, or three endoproteolytic cleavages. Six out of a possible total of eight forms of PA, resulting from incomplete cleavages at the three cleavage sites, have been identified and sequenced (Fig. 2).

Proteolytic Activity. Fig. 3 shows that PA was able to hydrolyze insulin A and B chain, gelatin, recombinant interleukin 2, and, to a lesser extent, myoglobin, ovalbumin, and fibrinogen. The degree of digestion was estimated in each HPLC chromatogram (Fig. 3) by the appearance of new peaks and the disappearance of the parent substrate after PA digestion. Only insulin B appeared to be completely digested. Hydrazinolysis performed with each PA/protein digestion mixture indicated that the new carboxyl-terminal residues after digestion were hydrophobic residues such as tyrosine, leucine, valine, phenylalanine, and basic residues of lysine, arginine, and histidine. Hence, PA possesses the same substrate specificity as chymotrypsin and trypsin.

The hydrolytic activity of PA was also studied with synthetic substrates in 0.1 M Tris·HCl, 0.1 M $CaCl_2$ (pH 8.0) at 25°C. PA exhibited a very low but detectable activity

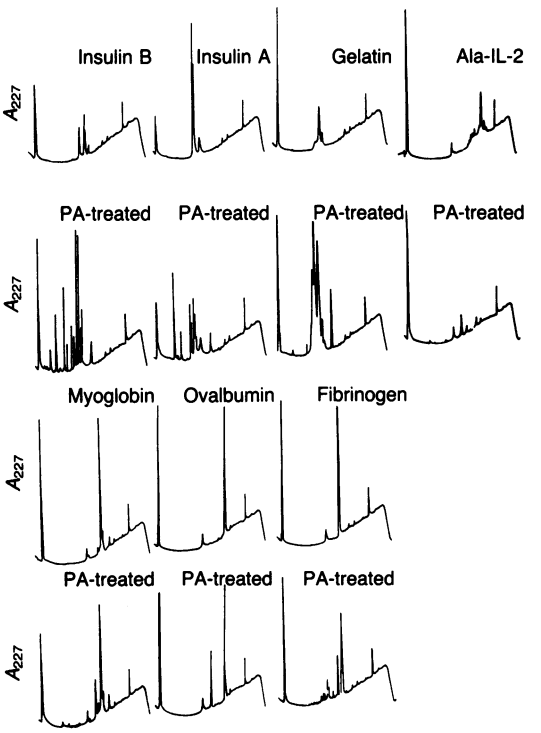

FIG. 3. Elution profiles of various proteins and their PA digestion products on reverse phase HPLC. The top panels represent the control experiments when the proteins were eluted on a Vydac C_4 column with the same gradient used to separate the digestion products of these proteins after PA treatment. The extent of PA digestions was highlighted by the appearance of newly generated peptide peaks shown in the bottom panels. A linear gradient of 0–80% (vol/vol) buffer B in 60 min was used.

toward Bz-Tyr-OEt, *N*-Bz-Arg-NHNpt, *N*-Suc-Phe-NA, and Tos-Arg-OMe. The enzyme hydrolyzed the synthetic chymotrypsin and trypsin *p*-nitroanilide ester substrates, namely, *N*-Suc-Ala-Ala-Pro-Phe-*p*-NA and *N*-Bz-Arg-*p*-NA, at a rate of 9.21 μM per min per mg and 1.98 μM per min per mg, respectively. A K_m value of 15.3 mM and a k_{cat} of 0.075 s^{-1} were obtained with *N*-Suc-Ala-Ala-Pro-Phe-*p*-NA as the substrate. The chymotrypsin type of activity of PA toward this substrate was about 200 times less than that of chymotrypsin (as measured by k_{cat}). The k_{cat}/K_m values for chymotrypsin and PA were 2.18×10^5 $M^{-1}S^{-1}$ and 4.88 $M^{-1}S^{-1}$, respectively. Protease inhibitors such as $PhMeSO_2F$, DFP, Tos-$PheCH_2Cl$, aprotinin, leupeptin, soybean trypsin inhibitor, as well as Zn^{2+} and spermidine when preincubated with PA for 16 hr at 25°C, were found to inhibit the enzymatic activity of PA. At 2 mM concentrations, $PhMeSO_2F$ and DFP gave an average of 90% inhibition of PA activity with *N*-Suc-Ala-Ala-Pro-Phe-*p*-NA, whereas the same concentration of Zn^{2+} completely abolished this chymotrypsin type of activity of PA.

DISCUSSION

We have determined the complete amino acid sequence of human PA. PA is a single polypeptide chain of 240 amino acids with a calculated M_r of 26,496 for the peptide portion. A potential polysaccharide side chain has been located on asparagine-45 with a sequence of Asn-Lys-Ser consistent with that found for other N-linked glycoproteins. Serine-69, threonine-70, and serine-71 are possible sites for O-linked carbohydrate side chains, because these three residues (69–71) gave significantly lower yields from Edman degradation when compared to that of serine-80, which appears later in the sequence. Comparisons of PA amino acid sequence with the published sequences of serine proteases show a very strong homology between PA and the various enzymes in the kallikrein family (Fig. 4). The homology is observed throughout the entire molecule, suggesting that their three-dimensional structures are very similar. The sequence identity of PA with kallikrein is 57% and with γ-nerve growth factor it is 56%. Less homology was seen with other serine proteases, including tonin (54%), epidermal growth factor-binding protein (53%), α-nerve growth factor (51%), trypsin (42%), chymotrypsin (35%), and *Streptomyces griseus* protease B (26%). The extensive homologies suggest that these proteases are descendants of a common ancestral gene.

Ten half-cystine residues are located in PA and occupy homologous positions to those of other serine proteases. Based on the known disulfide arrangements for trypsin, chymotrypsin, and γ-nerve growth factor, it is reasonable to assume a similar geometry for the five disulfide bonds of PA as a subset of the six disulfides present in bovine trypsin. Thus, cysteine-7 is linked to cysteine-152 (corresponding to residues 13 and 143 in trypsin); cysteine-26 to cysteine-42 (residues 31 to 47 in trypsin), which forms the histidine loop

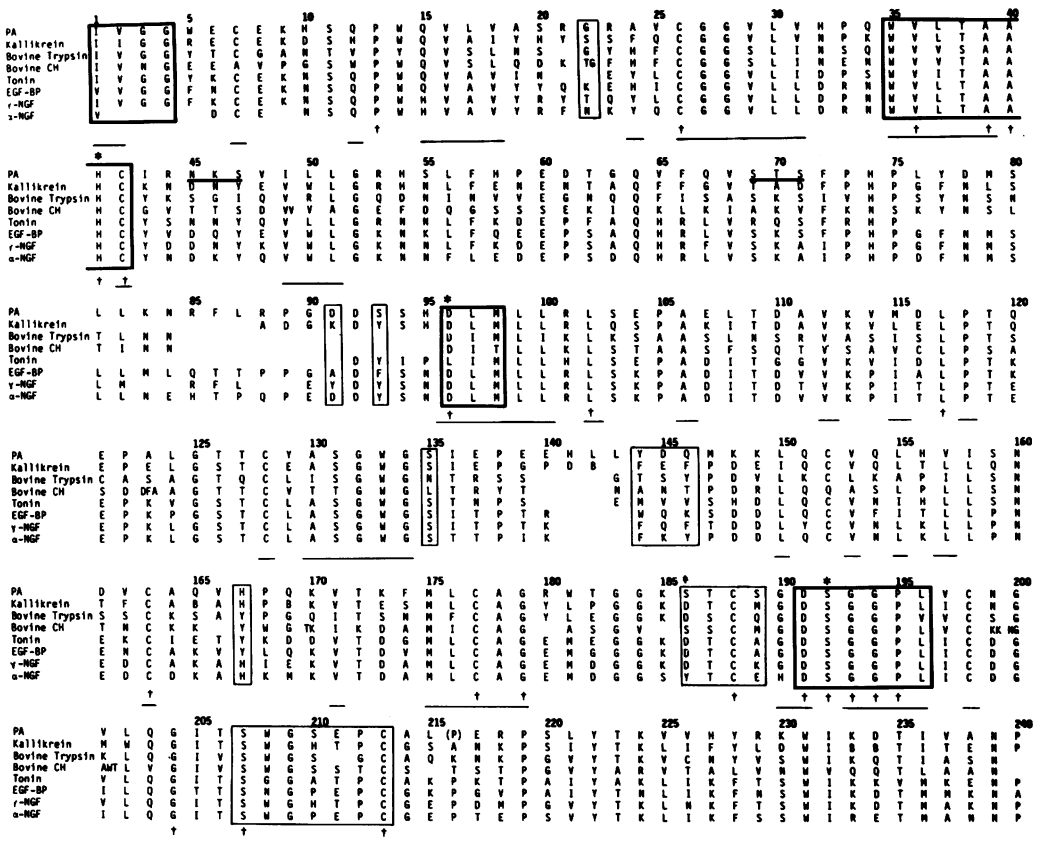

FIG. 4. Comparison of amino acid sequence of PA with other serine proteases including kallikrein (14), trypsin (15), chymotrypsin (15), tonin (16), epidermal growth factor-binding protein (17), γ and α subunit of 7 S nerve growth factor (18, 19). Gaps have been placed to maximize the homology. Heavily boxed areas contain residues neighboring the vicinity of the active sites of all serine proteases and are highly homologous. Areas that are lightly boxed indicate regions believed to line the substrate binding pocket (20). Residues underlined, numbering 45 and 69–71 of PA, represent potential attachment sites for carbohydrate. Columns of residues underlined are known to be internal and inaccessible to water molecules in the three-dimensional structures of α-chymotrypsin, trypsin, and elastase (21).

*Active site residues in PA, histidine-41, aspartic acid-96, and serine-192 are the components of the "catalytic triad" of serine proteases (22).

†Invariant residues conserved in all known serine proteases including human complement factor B (23) and bovine prothrombin (23).

‡Aspartic acid and serine residues at position 186 (numbering according to PA) determine the substrate specificity of trypsin and chymotrypsin, respectively.

responsible for proper orientation of histidine residue in the active site; cysteine-128 to cysteine-198 (residues 122 to 189 in trypsin); cysteine-163 to cysteine-177 (residues 154 to 168 in trypsin), which forms the methionine loop; and cysteine-188 to cysteine-213 (residues 179 to 203 in trypsin), which forms the serine loop linking together the primary and secondary binding sites (Fig. 2).

PA has been demonstrated to exhibit proteolytic activity similar to that of chymotrypsin and trypsin, and this enzymatic activity has been shown not to be due to contaminating enzymes (8). Thus, PA possesses similar proteolytic properties as those of $\overline{C1}$ esterase (24), tonin (16), and *Streptomyces griseus* protease B (25). Furthermore, the inactivation of PA by active-site directed reagents of serine proteases, such as $PhMeSO_2F$ and DFP, implies that PA is a serine protease with a catalytic apparatus similar to that of other serine proteases. The active site serine residue, serine-192, and the structurally adjacent histidine-41 and asparagine-96 residues of the charge–relay system are conserved as in all other serine proteases. The serine residue at position 186, which is located six residues before the active site serine-192, is in a homologous position to serine-189 in chymotrypsin that is believed to determine the substrate specificity for certain hydrophobic residues. The presence of a serine residue at this position, instead of aspartic acid residue as in trypsin, helps to explain the characteristics of PA that are similar to chymotrypsin. The additional ability of PA to hydrolyze bonds on the carboxyl side of basic residues may depend on the participation of carboxyl groups of glutamic acid residues in the binding of the substrate (12). Some possible candidates near the active site, in a position that could interact with the positive charges of the substrate, are glutamic acid-137, -139, and -140. In addition, glutamic acid-211, which is situated in the secondary binding site of PA, might also be involved.

All but 1 of the 29 "invariant" amino acid residues of the serine proteases (15) are conserved in the PA sequence. Leucine-155 and proline-161 in chymotrypsin are situated at the beginning and end of a conserved β-pleated stretch. In the corresponding positions in PA, leucine also occupies position 150, but proline is substituted by histidine at position 156. This may alter the secondary structure in this portion of the PA molecule. Interestingly, histidine residue is also located at this position in tonin (16). Identical sequences to those of other serine proteases are also retained in the two regions for the substrate-binding site of PA, including the primary binding site at residues 190–195 (Gly-Asp-Ser-Gly-Gly-Pro) and the secondary binding site corresponding to residues 207–209 (Ser-Trp-Gly).

In eukaryotic multicellular organisms, the serine proteases are synthesized as single chain precursors, and subsequent activation involves proteolytic cleavage of the peptide bond preceding the residue at position 16. The α-amino group of residue 16 forms a salt bridge following activation with the side chain of aspartic acid residue next to the active center serine (26). In PA, the preservation of the active-site residues, histidine-41, aspartic acid-96, and serine-192, as well as the amino-terminal hydrophobic sequence Ile-Val-Gly-Gly, lends strong support to the notion that PA is also synthesized as a single polypeptide precursor. Furthermore, the invariant sequence of Gly-Trp-Gly found in most serine proteases (residues 140–142 in chymotrypsin) also occurs in homologous positions in PA (residues 132–134). This sequence is found to lie at the beginning of the autolysis loop in chymotrypsin and is believed to serve a critical function in the conversion of a flexible zymogen structure to a fixed rigid orientation in the activated protease (27).

Analysis of the amino-terminal sequence of purified PA has defined the location of three endoproteolytic cleavages. These cleavages may be autocatalytic like those of other serine proteases. Two of the cleavage sites in PA, namely, following lysine-148 and arginine-85, occupy homologous positions to those of γ-nerve growth factor (18) and are situated on opposite sides of the substrate binding site region between different β-strands of the molecule.

The question still remains regarding the *in vivo* substrate of PA. Since the initial report in 1962 by Huggins and Neal (28) on the presence of "fibrinolytic" activity in human and canine prostatic fluid, various proteolytic enzymes of human seminal plasma have been characterized, including plasminogen activators, a neutral protease (seminin), and a pepsin type of enzyme (29). However, the physiological substrates and functions of these enzymes have not been determined. Whether the physiological role of PA is connected to liquefaction of seminal clot or to the fibrinolytic activity in semen, acts as a kininogenase (30), or is involved in the proteolytic processing of biologically active polypeptides remains to be studied.

Note. Since the submission of this article, Lilja (31) reported that the structural protein of human seminal coagulum, the predominant seminal vesicle secreted protein, might be the physiological substrate for PA.

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