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Specific and Efficient Peptide Substrates for Assaying the Proteolytic Activity of Prostate-specific Antigen¹

Samuel R. Denmeade, Wei Lou, Janita Lövgren, Johan Malm, Hans Lilja, and John T. Isaacs²

The Johns Hopkins Oncology Center [S. R. D., W. L., J. T. I.], and James Buchanan Brady Urological Institute [S. R. D., J. T. I.], Johns Hopkins School of Medicine, Baltimore, Maryland 21231-1001; Department of Biotechnology, University of Turku, Turku, FIN-20520, Finland [J. L.]; and Department of Clinical Chemistry, Lund University, University Hospital, Malmö S-21401, Sweden [J. M., H. L.]

Abstract

Prostate-specific antigen (PSA) is a serine protease secreted by both normal prostate glandular cells and prostate cancer cells. The major proteolytic substrates for PSA are the gel-forming proteins in semen, semenogelin (Sg) I and II. On the basis of the PSA cleavage map for Sg I and II, a series of small peptides (*i.e.*, 7 amino acids) was synthesized and coupled at the COOH terminus to 7-amino-4-methyl coumarin. Using these fluorescently tagged substrates, K_m s and k_{cat} s were determined for PSA hydrolysis, and the substrates were also tested for activity against a panel of purified proteases. Previously, a variety of chymotrypsin substrates have been used to assay the enzymatic activity of PSA. The present studies have identified a peptide sequence with a high degree of specificity for PSA (*i.e.*, no detectable hydrolysis by chymotrypsin) and improved K_m s and k_{cat} s over previously used substrates. On the basis of these parameters, the best peptide substrate for PSA has the amino acid sequence HSSKLQ. Using PC-82 human prostate cancer xenografts and human prostate tissues, this PSA substrate was used to document that prostate cancer cells secrete enzymatically active PSA into the extracellular fluid but that once in the blood, PSA is not enzymatically active. On the basis of this information, it should be possible to use the HSSKLQ peptide as a carrier to target peptide-coupled prodrugs for selective activation within sites of PSA-secreting, metastatic prostate cancer cells and not within the blood or other nonprostatic normal tissues.

INTRODUCTION

Currently, there is no treatment that significantly prolongs survival in men with metastatic prostate cancer (1). Androgen ablation therapy, although of substantial palliative benefit, has had little impact on overall survival, and the mortality rate from prostate cancer has increased steadily since the introduction of this therapy in the 1940s (2). Androgen ablation

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²To whom requests for reprints should be addressed, at Johns Hopkins Oncology Center, Johns Hopkins School of Medicine, 422 North Bond Street, Baltimore, MD 21231-1001.

therapy eventually fails, because the metastatic prostate cancer within an individual patient is composed heterogeneously of clones of both androgen-dependent and -independent cancer cells (3). Due to these androgen-independent prostatic cancer cells, the patient is no longer curable using the numerous chemotherapeutic agents that have been tested over the past 30 years (1). Thus, there will be an estimated 41,000 deaths this year in the United States due to androgen-independent metastatic prostate cancer (2).

Standard antiproliferative chemotherapeutic agents may be ineffective against androgen-independent prostatic cancers because these cancers have a low proliferative rate. Berges *et al.* (4) demonstrated that the median daily proliferative rate of prostate cancer cells within lymph node or bone metastases was <3.0% per day. Newer agents are needed that target the >95% of prostate cancer cells within a given metastatic site that are not immediately proliferating. An example of such an agent is TG,³ a potent and selective inhibitor of the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase pump (5, 6). Inhibition of the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase pump by TG results in a sustained elevation of intracellular Ca²⁺, which induces programmed cell death/apoptosis of metastatic, androgen-independent human and rodent prostatic cancer cells in a proliferation independent manner (5–7). TG is also able to induce programmed cell death in proliferatively quiescent (*i.e.*, G₀) human prostate cancer cells in primary culture without recruitment into the proliferative cell cycle (8). Because of its unique cell proliferation-independent mechanism of cytotoxicity, TG could be a novel agent for the treatment of metastatic prostate cancer. However, TG itself is difficult to administer systemically without significant toxicity, because it is highly lipophilic, and its cytotoxicity is not prostate cancer cell specific.

As an approach to target cytotoxicity of TG specifically to metastatic prostate cancer, a prodrug could be developed consisting of a primary amine containing a TG derivative coupled via its amino group to form a peptide bond to a water-soluble peptide carrier (9). The peptide carrier may be designed so that the peptide linkage between the amino-TG derivative and the peptide is efficiently and specifically cleavable only by the proteolytic activity of PSA. PSA is a *M_r* 33,000 single-chain glycoprotein first characterized from human prostate tissue by Wang *et al.* (10). PSA is synthesized and secreted as a unique differentiation product of the prostatic glandular cells (10–12). No other normal cell types in the human secrete PSA in any significant level (10–12). Besides normal prostatic glandular cells, the only other cell type in males that secretes substantial amounts of PSA are prostate cancer cells (10–12). Low levels of PSA have also been detected in homogenates from normal breast tissue and breast cancer (13); however, serum levels of PSA are not detectable, even in women with breast cancers staining positive for PSA (14).

PSA is a serine protease with extensive sequence identity to the glandular kallikreins (11, 12, 15). However, unlike the glandular kallikreins that have a common trypsin-like activity, PSA has chymotrypsin-like substrate specificity (15–17). The major proteolytic substrates for PSA are the gel-forming proteins in freshly ejaculated semen, Sg I and II, which are

³The abbreviations used are: TG, thapsigargin; PSA, prostate-specific antigen; Sg, semenogelin; AMC, amino-4-methyl-coumarin; mAb, monoclonal antibody; ACT, α₁-antichymotrypsin.

synthesized and secreted by the seminal vesicles (11, 16, 18). The ejaculatory mixing of the secretions from the seminal vesicles and the prostate results in immediate formation of a loosely connected gel structure that entraps spermatozoa. Sg I and II are the major structural proteins in the gel, and enzymatically active PSA in the seminal fluid proteolytically generates multiple soluble fragments of Sg I and II (11). Lilja *et al.* previously described several PSA-specific cleavage sites within Sg I(16), and recently, additional cleavage sites were defined within both Sg I and II (19).

Previously, a variety of chymotrypsin substrates have been used to assay the enzymatic activity of PSA (12, 16, 20). These substrates have relatively high K_{mS} (*i.e.*, 1–2 mM), low k_{catS} for PSA, and poor specificity (*i.e.*, 10,000-fold more rapidly cleaved by chymotrypsin). The aim of the present study was to develop a peptide substrate for PSA with improved K_{mS} and k_{catS} , a high degree of specificity for PSA, and stability in serum so that it could be used as a carrier to specifically target TG prodrug activation by PSA-secreting prostatic tissue. On the basis of the PSA cleavage map for Sg I and II (19), small peptides were synthesized containing the amino acid sequence proximal to the PSA proteolytic sites and having 7-AMC coupled via a peptide bond to the carboxylic residue of the terminal amino acid. This allowed hydrolysis at the COOH-terminal residue to be followed kinetically by the increase in fluorescence due to release of the AMC. Kinetics parameters (*i.e.*, K_{mS} and k_{catS}) were determined for the proteolysis of these substrates by PSA, and activity against a panel of purified proteases was tested to identify the best substrate based on its efficacy and specificity for PSA.

MATERIALS AND METHODS

Materials

PSA was purified from human seminal plasma as described previously (15). Recombinant hK2 was generated as described previously (21). Plasmin, urokinase, tissue plasminogen activator, human plasma, and porcine pancreatic kallikrein and ACT were obtained from Calbiochem (La Jolla, CA); all other proteases were obtained from Sigma Chemical Co. (St. Louis, MO). All of these proteases were tested separately with the appropriate standard substrate to document that the proteins were in the active form. Mouse monoclonal IgG anti-PSA antibodies H117 and 5A10 were described previously (21–23). Mouse serum was obtained from East Acres Biologicals (South Bridge, MA). Goat and calf sera were obtained from Life Technologies, Inc. (Grand Island, NY). Rat serum and mouse plasma were obtained in-house from sacrificed animals. Human serum was obtained from patients with prostate cancer who had PSA values between 165 and 689 ng/ml by Tandem-R assay. Human control serum was obtained from male volunteers without prostate cancer with serum PSA levels <0.5 ng/ml by Tandem-R assay.

Cell Line and Collection of PSA

PC-82 androgen-responsive human prostate cancer xenografts were maintained by serial passage in nude mice (Charles River Breeding Laboratories). The origins and characteristics of this xenograft have been described previously (24–26). Blood from tumor-bearing animals was obtained by retro-orbital sinus puncture under metofane anesthesia

(Mallinkrodt, Mundelein, IL). To determine the proportion of PSA in the extracellular fluid of PC-82 tumors that are enzymatically active, freshly removed PC-82 tumors (200–300 mg) were wetted with ~100 μ l RPMI, and then after 10 min, fluid was wicked off the surface of the tumor and collected for additional analysis after determining the PSA concentration by Tandem-R assay (Hybritech, San Diego, CA). The PSA in the extracellular fluid was then concentrated by immunoprecipitation and assayed for its enzymatic activity as described below. PC-82 homogenates were made using a hand-held homogenizer to liquefy tumors (300–600 mg) in 1 ml of PSA assay buffer [50 mM Tris and 0.1 M NaCl (pH 7.8)].

To determine the activity of PSA in the extracellular fluid of normal human prostate tissue and prostate cancers, radical prostatectomy specimens were obtained, and normal peripheral prostate tissues and cancers were dissected separately by a pathologist. These tissues were then wetted with RPMI at room temperature as described above, and extracellular fluid was collected. PSA concentrations of this tissue extracellular fluid were determined using the Tandem-R assay, and then samples were diluted with PSA assay buffer to a final PSA concentration of 4 μ g/ml prior to direct enzymatic assay.

PSA Substrates

PSA substrates were custom synthesized by Enzyme Systems Products (Dublin, CA), and, after high-performance liquid chromatography separation and purification to >95%, the molecular weights were confirmed by mass spectroscopy. All substrates had AMC attached via an amide bond to the carboxyl group of the COOH-terminal amino acid. The NH₂ terminus of the substrates was protected by coupling to a 4-morpholinecarbonyl blocking group (formula weight 115). 4-Morpholinecarbonyl was chosen as the NH₂-terminal blocking group because of its stability and to enhance substrate solubility. Substrates were first dissolved in distilled H₂O (5 mM) and then diluted into PSA assay buffer. The glutamine-AMC (Q-AMC) substrate had the NH₂ terminus and side chain amine unprotected and was obtained from Bachem (Torrance, CA)

Kinetic Analysis of Substrate Hydrolysis by PSA

Substrate hydrolysis was studied by measuring fluorescence change secondary to AMC release using a Fluoroskan II 96-well fluorometric plate reader (ICN Biomedicals, Costa Mesa, CA; excitation, 355 nm; emission, 460 nm) connected to a Macintosh computer. Data were collected and analyzed using Deltasort III software (Biometallics, Princeton, NJ). All reactions were performed at room temperature by the addition of substrate and proteases into PSA assay buffer, described previously, in a final volume of 200 μ l. The results were determined from the initial linear increase of fluorescence and were expressed as the pmol of AMC released per min based on comparison to a standard curve of the fluorescence of known amounts of 7-AMC (*i.e.*, fluorescence was linear from 20 to 3000 pmol of AMC). The data were analyzed by Lineweaver-Burke reciprocal plots to determine the Michaelis-Menten constant (*i.e.*, K_m), expressed as the amount of substrate needed to saturate half of the enzyme, and the catalytic rate constant (*i.e.*, k_{cat}), expressed as the amount of substrate converted to product per time per amount of enzyme.

PSA Immunoprecipitation

PSA levels in the serum and extracellular fluid from PC-82 tumor-bearing animals were measured by Tandem-R assay, and samples were then diluted to 600–1000 ng PSA/ml of PSA buffer. After incubation with 100 μ l of 50% protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) beads for 30 min at 4°C, the mixture was centrifuged at 10,000 rpm for 5 min to preclear the supernatant. To the resultant supernatant was added mouse mAb H117, which recognizes both PSA and glandular kallikrein (hK2; Ref. 21), to a final concentration of 5 μ g/ml. Samples were rotated at 4°C overnight, and then 100 μ l of protein A-Sepharose were added with rotation for 3 h at 4°C. The mixture was centrifuged to pellet the captured PSA, and then the pellets were washed three times with PSA buffer at room temperature. Aliquots of the washed pellets were then analyzed for enzymatic activity with PSA substrates in 96-well plates by measuring fluorescent change over time. Analysis of supernatants by Tandem-R assay after immunoprecipitation demonstrated >95% capture of PSA (final concentration, <5 ng/ml). Purified PSA from human seminal plasma was used as a positive control and immunoprecipitated as above. The specificity of captured PSA was determined by Western blotting and by inhibition of enzymatic activity of immunoprecipitated PSA by a second mouse mAb, mAb 5A10 (10 μ g/ml), that is specific for free PSA (*i.e.*, uncomplexed to serum protease inhibitors), does not cross-react with hK2, and inhibits the enzymatic activity of PSA (21–23).

Western Blotting

The protein A-Sepharose-immunoprecipitated pellets were suspended in loading buffer [50 mM Tris (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromphenol blue, and 10% glycerol], and the samples were heated to 100°C for 5 min. The Sepharose beads were spun down, and aliquots of the supernatant containing 25 ng of PSA (*i.e.*, based on pre-pelleted determination of PSA concentration by Tandem-R assay) were separated on a 12% SDS-PAGE gel. The proteins were then electroblotted to Immobilon membranes (Millipore, Bedford, MA). The membranes were processed by standard Western blot procedures and incubated with a polyclonal rabbit antihuman PSA antibody (DAKO Corp., Carpinteria, CA) diluted 1:1000. The immune complexes were visualized with the enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL) according to the manufacturer's specifications.

RESULTS

Properties of PSA Substrates

In freshly ejaculated semen, the proteolytic activity of PSA is directed mainly against the major gel-forming proteins Sg I and II. A map of PSA cleavage sites within the Sg proteins has been generated (16, 19), and peptides of seven amino acids representing the sequence proximal to the PSA cleavage site (P₇ – P₁) were synthesized with AMC attached to the COOH terminus of the P₁ amino acid in the P₋₁ position. A series of 12 sequences obtained from the Sg cleavage map were synthesized and tested initially as substrates for purified PSA and a panel of other purified extracellular proteases (Table 1). To allow valid comparisons, each assay contained 200 pmol of the particular protease and 0.2 mM concentration of the particular substrate (*i.e.*, 40,000 pmol/200 μ l of assay volume). Those

substrates containing tyrosine in the P₁ position were hydrolyzed efficiently by PSA; however, proteolysis rates were 10–20-fold higher with chymotrypsin and elastase. Substrates with glutamine in the P₁ position were less efficiently hydrolyzed but demonstrated better specificity for PSA (Table 1).

These assays identified four groups of substrates based on PSA activity and specificity. On the basis of these kinetic parameters and specificities of the substrates, four groups of PSA substrates were identified. The first group consisted of those sequences (KGISSQY, SRKSSQY, and GQKGQHY) with the highest PSA activity but that were even better substrates for other serine proteases, such as chymotrypsin and elastase. The second group (EHSSKLQ, QNKISYQ, ENKISYQ, and ATKSKQH) had moderate PSA activity but showed greater specificity for PSA. The third group (KGLSSQC and LGGSQQL) also had moderate PSA activity but were less specific for PSA than was the second group. The fourth group (QNKQHYQ, TEERQLH, and GSFSIQH) had little activity with any of the proteases tested. All substrates lacking tyrosine at the cleavage site had undetectable activity with those serum protease such as thrombin, plasmin, and urokinase that have trypsin-like specificity for cleavage primarily after arginine residues. PSA is a member of the kallikrein family of proteases (*i.e.*, PSA is hK3) and shares substantial sequence homology with human plasma kallikrein (hK1) and human glandular kallikrein (hK2; Ref. 21). hK1 and hK2 also have trypsin-like substrate specificity (21) but demonstrated no detectable activity with the majority of substrates (Table 1).

On the basis of these preliminary studies concerning solubility and specificity, a selected subset of substrates were studied further to determine their K_m and k_{cat} kinetic parameters (Table 2). Because the k_{cat} reflects the efficiency of the conversion of the substrate to products per enzyme whereas the K_m reflects the concentration dependence for enzymatic activity, higher $k_{cat}:K_m$ ratios denote better substrates, and these ratios were calculated to compare the various substrates.

Stability of PSA Substrates in Sera

Because the prodrug would need to be given systemically via the blood, additional analyses were performed to determine the stability of the substrates in sera from a variety of species (Table 3). Hydrolytic activity varied widely between substrates and between sera. All of the substrates with tyrosine in the P₁ position and two with glutamine (ENKISYQ and QNKISYQ) were hydrolyzed to a greater extent than the others, especially in mouse and fetal calf sera, whereas the remaining substrates showed good stability in human sera for periods up to 24 h.

Effect of Sequence Length on PSA Activity

The substrate EHSSKLQ-AMC was chosen from among the others for additional characterization based on its PSA activity, its specificity for hydrolysis by PSA, and its relative stability in a variety of sera. On the basis of this starting sequence, shorter sequence lengths were then synthesized starting from the COOH terminus and analyzed for activity with PSA and other purified proteases (Table 1). In general, the shorter sequence length maintained specificity for proteolysis by PSA when compared to the panel of other purified

extracellular proteases. The six-amino acid peptide, HSSKLQ, was even a better substrate for PSA without sacrificing any specificity. Next, K_m s and k_{cat} s were determined for each sequence length (Table 2). From this group, the six-amino acid sequence HSSKLQ-AMC had the lowest K_m . This peptide also maintained a high degree of specificity for PSA as well as good stability in sera (Table 3). The four-amino acid sequence SKLQ-AMC, although having a higher K_m than HSSKLQ-AMC, had an equivalent $k_{cat}:K_m$ ratio and serum stability. However, this shorter peptide was less water soluble than the histidine-containing HSSKLQ. Because of comparable activities (*i.e.*, $k_{cat}:K_m$ ratios) between the four- and six-amino acid sequences, the five-amino acid sequence was not synthesized. The three-amino acid sequence KLQ-AMC had detectable activity at 5 mM concentration, whereas the shorter one- and two-amino acid substrates had no detectable activity at 5 mM. Substrates were not evaluated at concentrations >5 mM because of poor solubility in aqueous buffer. On the basis of these combined data, the best substrate for PSA analysis is HSSKLQ-AMC.

PSA Activity in the PC-82 Human Xenograft

On the basis of its $k_{cat}:K_m$ ratio, relative specificity, serum stability, and water solubility, the HSSKLQ-AMC substrate was used to analyze the enzymatic activity of PSA in the PC-82 xenograft model. PC-82 is an androgen-responsive tumor and is one of the few human prostate cancers that continues to secrete PSA during serial xenograft passage into nude mice (25). Sera from mice bearing 300–500-mg PC-82 tumors contain elevated levels of Tandem-R-detectable PSA (200–400 ng/ml).

Initially, homogenates of PC-82 tumors were made, and the PSA concentration in these homogenates was determined using the Tandem-R assay. The enzymatic activity of these homogenates was then assayed using the substrate HSSKLQ-AMC, and the activity was compared to that of an equal amount of PSA purified from human seminal fluid (Fig. 1A). These PC-82 homogenates hydrolyzed the substrate at a 6-fold higher rate than the purified PSA, and most of this activity (*i.e.*, $>82 \pm 2\%$) was not inhibited by the addition of anti-PSA antibody 5A10 (Fig. 1A). These studies indicated that the substrate HSSKLQ-AMC, although not a substrate for a variety of purified extracellular proteases, could be hydrolyzed by a combination of intracellular proteases.

To further clarify this point, the family of peptide substrates based on the EHSSKLQ sequence was assayed for activity for the intracellular proteases cathepsin B, C, and D and esterase (Table 4). As the sequence length became incrementally shorter, the rate of hydrolysis by cathepsin B and D increased, whereas cathepsin C and esterase had no detectable activity with all of the sequences tested (Table 4). The single amino acid substrate, glutamine-AMC, was not hydrolyzed efficiently by any of these intracellular proteases.

To allow the best substrate (*i.e.*, HSSKLQ-AMC) to be useful for assaying the PSA enzymatic activity in homogenates of whole tumors, an immunoprecipitation step was used to isolate enzymatically active PSA that was free of contamination by other intracellular proteases. PSA can be immunoprecipitated with mAb H117, a mAb that recognizes a shared epitope of PSA and hK2 outside their catalytic centers (21), and therefore, the immunoprecipitate does not inhibit the enzymatic activity of either enzyme. For example,

when purified PSA from human seminal plasma is immunoprecipitated in this manner, it continues to have enzymatic activity when incubated with the PSA substrate HSSKLQ-AMC (Fig. 1B). Substrate hydrolysis is specific for PSA, because hK2 is unable to cleave the PSA substrate and because the proteolytic activity can be stopped immediately upon the addition of mAb 5A10, a mAb that only recognizes free PSA and not hK2 and inhibits the enzymatic activity of PSA (Ref. 22; Fig. 1B). These results validate that the immunoprecipitation protocol used can effectively capture enzymatically active PSA. Using this immunoprecipitation protocol and the HSSKLQ-AMC substrate at 0.4 mM concentration, PSA enzymatic activity was demonstrated to increase linearly with increasing amounts of purified seminal plasma PSA (*i.e.*, linear range, 50–1000 ng of PSA; Fig. 1C). The lower limit of detection of PSA enzymatic activity with the HSSKLQ-AMC substrate (*i.e.*, >0.1 pmol of AMC released/min) is 50 ng of total PSA per assay (Fig. 1C).

Using this validated immunoprecipitation protocol, PSA was immunoprecipitated with mAb H117 from homogenates made from PC-82 tumors and assayed for enzymatic activity using the HSSKLQ-AMC substrate (Fig. 1D). Like the enzymatic activity of the purified seminal plasma PSA immunoprecipitate, the enzymatic activity of the PSA immunoprecipitate from the PC-82 tumor homogenates was inhibited by >98% by the neutralizing mAb 5A10 (Fig. 1D). PSA immunoprecipitated from these PC-82 tumor homogenates possessed $70 \pm 5\%$ of the enzymatic activity of similar concentrations of immunoprecipitated PSA purified from seminal plasma.

The PC-82 homogenate consists of PSA from both intracellular and extracellular compartments. To analyze the enzymatic activity of PSA only in the extracellular fluid, microcapillary tubes were used to wick fluid off the surface of freshly removed PC-82 tumors. The PSA concentration in this fluid was determined by Tandem-R assay, and after dilution to 1 $\mu\text{g}/\text{ml}$ in PSA assay buffer, the PSA was immunoprecipitated according to the previously described protocol. The immunoprecipitates were then assayed for PSA enzymatic activity using the HSSKLQ-AMC substrate (0.4 mM; Fig. 2A). As expected, the enzymatic activity of the PSA immunoprecipitated from the PC-82 tumor extracellular fluid was also inhibited by mAb 5A10 (Fig. 2A). PSA immunoprecipitated from the PC-82 extracellular fluid possessed $66 \pm 4\%$ of the enzymatic activity of similar concentrations of immunoprecipitated PSA purified from seminal fluid.

PSA was also immunoprecipitated from the pooled sera of animals bearing PC-82 tumors and assayed for enzymatic activity using the HSSKLQ-AMC substrate (Fig. 2B). The lack of PSA enzymatic activity in serum (Fig. 2B) is consistent with previous studies showing that PSA in human serum is enzymatically inactivated by interaction with the major extracellular protease inhibitors ACT and α_2 -macroglobulin (21). These inhibitors are present in a 10^4 – 10^5 -fold molar excess to PSA in serum (15, 22, 23). PSA forms a 1:1 molar ratio complex with ACT by splitting the reactive center of the inhibitor, thus forming a M_r 90,000 stable PSA-ACT covalent complex (Ref. 26; Fig. 2C, Lane 2).

Western blot analysis of the PSA in the sera of PC-82-bearing animals demonstrated that the majority of the immunoprecipitable PSA is in the M_r 90,000 PSA-ACT complex (Fig. 2C, Lane 3), which is enzymatically inactive, as shown in Fig. 2B. In the immunoprecipitated

extracellular fluid obtained from the PC-82 tumors, PSA can be found in two forms on Western blot, $\approx 25\%$ being the M_r 90,000 PSA-ACT complex and the remaining 75% in the M_r 33,000 uncomplexed, free form of PSA (Fig. 2C, Lane 4). The M_r 33,000 free PSA is the enzymatically active form that is capable of hydrolyzing the HSSKLQ-AMC substrate Fig. 2A.

Previously, it has been demonstrated that the complex formed between PSA and α_2 -macroglobulin does not result in the complete inactivation of PSA, because smaller substrates can still be hydrolyzed by the PSA- α_2 -macroglobulin complex (21). The PSA- α_2 -macroglobulin complex is not immunoreactive with PSA antibodies, and therefore, this complex would not be captured during the immunoprecipitation of PSA from the sera of PC-82 tumor bearing animals. To determine whether PSA can be inhibited completely by the full complement of serum protease inhibitors present in mouse blood, plasma was obtained from nude mice, and purified, enzymatically active human seminal plasma PSA was added to a concentration of 5 $\mu\text{g/ml}$. Samples were incubated at 37°C for 24 h and then assayed directly for PSA activity using the HSSKLQ-AMC substrate. In this experiment, there was no difference in the rate of hydrolysis of the HSSKLQ-AMC substrate in the PSA-spiked mouse plasma *versus* control plasma ($n = 6$, data not shown), demonstrating that the PSA was inhibited completely by the mouse plasma. The addition of the anti-PSA mAb 5A10 resulted in no further inhibition of hydrolysis in both PSA-containing and control plasma.

PSA Activity in Human Prostate Tissue and Serum

Extracellular fluid from normal and malignant prostate tissue was assayed for its PSA concentration using the Tandem-R assay. The extracellular PSA concentrations ranged from 30 to 110 $\mu\text{g/g}$ for the normal prostate tissue ($n = 8$; mean = $13.9 \pm 6.6 \mu\text{g/ml}$) and 20–110 $\mu\text{g/g}$ for the prostate cancers ($n = 7$; mean = $9.9 \pm 6.3 \mu\text{g/ml}$). Individual samples were diluted to a final concentration of 4 $\mu\text{g/ml}$ and assayed directly for PSA enzymatic activity using the HSSKLQ-AMC substrate. To determine the percentage of PSA enzymatic activity in these samples, the anti-PSA mAb 5A10, which inhibits >99% of the enzymatic activity of PSA from purified human seminal plasma, was added to a final concentration of 50 $\mu\text{g/ml}$. The percentage decrease in slope upon addition of mAb 5A10 *versus* control samples was then calculated. For normal prostate tissue, the mAb 5A10 inhibited $78.0 \pm 2.4\%$ ($n = 16$) of the activity in the extracellular fluid, whereas in the fluid from prostate cancers, the mAb 5A10 inhibited $89.1 \pm 2.0\%$ ($n = 13$) of the activity. These results demonstrate that PSA is enzymatically active in the extracellular fluid obtained from both normal and malignant prostate tissues and that PSA is responsible for the majority of HSSKLQ-AMC substrate hydrolysis in the extracellular fluid from these tissues.

In additional studies, the HSSKLQ-AMC substrate was used to assay without immunoprecipitation the enzymatic activity of PSA in the sera of four men with prostate cancer and elevated Tandem-R-detectable serum PSA levels ranging from 165 to 689 ng/ml. Using these serum samples, no hydrolysis of the HSSKLQ-AMC substrate was detected (*i.e.*, <0.1 pmol AMC released/min).

DISCUSSION

The aim of the present study was to identify a PSA-specific peptide substrate that can be used as a carrier for targeted delivery of cytotoxic agents to sites of metastatic prostate cancer. Substrates for PSA include Sg I and II (16, 19), the extracellular matrix components fibronectin (16) and laminin (27), the insulin-like growth factor binding proteins (28), the single-chain form of urokinase-type plasminogen activator (29) and parathyroid hormone-related protein (30). PSA, through its ability to degrade extracellular matrix components, may also play a role in tumor cell invasion and metastases (27). From a map of PSA cleavage sites within Sg I and II (16, 19) we have generated a series of PSA substrates. On the basis of assays comparing the proteolysis of these substrates by a panel of purified proteases, determination of kinetic parameters, and measurements of stability in sera, one substrate, HSSKLQ-AMC, was selected for further analysis. This substrate has high specificity for PSA, and it is stable in human sera, including sera from men with elevated PSA levels and human plasma. The K_m for this substrate is 470 μM , which is a 3–5-fold improvement over the K_m s for previously used PSA substrates, which have been primarily chymotrypsin substrates with K_m s for PSA in the 1.3–2 mM range (15, 17, 20).

Besides having an improved K_m and k_{cat} , the HSSKLQ substrate is not measurably hydrolyzed by a panel of purified extracellular proteases, including chymotrypsin and trypsin. The only other protease with any significant ability to hydrolyze this substrate is the intracellular lysosomal cysteine protease, cathepsin B. In normal cells, lysosomal proteases are secreted extracellularly as inactive zymogens that are then taken up by the cell and delivered to its lysosomal compartment via the mannose-6-phosphate receptor pathway (31). Several laboratories have reported an association between increased plasma membrane association and/or activity of cathepsin B and either malignant conversion or enhanced metastatic capability (32, 33). Cathepsin B is not present in significant amounts in the extracellular fluid of normal cells but appears to be substantially elevated in the extracellular fluid surrounding neoplastic cells, including prostate cancer cells (34, 35). The proteolysis of HSSKLQ-AMC by cathepsin B demonstrates that this substrate is not entirely PSA specific. However, proteolysis by active cathepsin B secreted by cancer cells may result in additional, selective activation of the prodrug within sites of metastatic prostate cancer.

Besides cathepsin B, HSSKLQ-AMC is not a substrate for a large panel of purified extracellular proteases. The HSSKLQ-AMC substrate, however, is hydrolyzed readily by tumor homogenates, indicating that intracellular proteases, presumably from lysosomes, are capable of cleaving this substrate. The nonspecific hydrolysis of the HSSKLQ-AMC substrate by intracellular proteases requires that an immunoconcentration step be used prior to assaying the enzymatic activity of PSA in tumor homogenates. This also requires that an incubation of the immunoconcentrated proteins with a specific PSA-neutralizing antibody be performed to ensure that the enzymatic activity seen is due to the PSA and not due to hydrolysis by other intracellular or extracellular proteases.

In men with prostate cancer, PSA levels in the blood can exceed 1000 ng/ml. This serum PSA is derived from the PSA in the extracellular fluid. Upon escape into the extracellular compartment, PSA is initially enzymatically active, as demonstrated by the PC-82 and

human prostate cancer results presented. Extracellular prostate cancer fluid can hydrolyze the HSSKLQ-AMC substrate and can form complexes with the extracellular protease inhibitor, ACT. Once in the serum, however, PSA has no assayable enzymatic activity due to its complexing with ACT and α_2 -macroglobulin (15, 23). A small portion of PSA occurs in a free, noncomplexed (M_r 33,000) form that is apparently enzymatically inactive due to its inability to form complexes with the excess protease inhibitors (23). The enzymatic inactivity of this M_r 33,000 form of PSA in the blood is supported by our data showing that little to no hydrolysis of the PSA substrate HSSKLQ-AMC occurred in the blood of men with elevated PSA levels. PSA immunoprecipitated from the blood serum of PC-82-bearing animals also had no enzymatic activity, and Western blot analysis demonstrated that the majority of the serum PSA is found in the M_r 90,000 PSA-ACT complex.

The small amount of free PSA in the serum most likely represents either the PSA proenzyme form or PSA that has been cleaved internally. PSA is produced as a proenzyme. Prior to secretion, a 17-amino acid hydrophobic signal sequence is removed from the NH_2 terminus to produce an enzymatically inactive proenzyme (36). The inactive proenzyme has a seven-amino acid activation peptide that must be released through cleavage at the ultimate arginine preceding the NH_2 -terminal isoleucine in the mature form of PSA to gain enzymatic activity (36). This cleavage site predicts that a trypsin-like protease converts the zymogen into enzymatically active PSA, and indeed, trypsin has been shown to activate recombinant pro-PSA produced in *Escherichia coli* (37). PSA secreted by PC-82 into the extracellular fluid is processed correctly, because the PSA isolated from this fluid is enzymatically active. These results demonstrate that PSA is activated before entering the serum and not as a result of processing by a protease present within the blood or contributed by any of the other accessory sex glands in the male genital tract. The enzymatic activity in the extracellular fluid indicates that the processing enzyme must be present either within the prostate cancer cells themselves or in the extracellular fluid. If it is in the extracellular fluid, this processing enzyme is produced either by the PC-82 cells themselves or by the surrounding tissue. To resolve this issue, conditioned media from primary cultures of PC-82 prostate cancer cells established in serum-free defined media to prevent stromal cell growth are being assayed using the AMC-tagged seven-amino acid PSA propeptide sequence as a substrate for this PSA-processing enzyme.

In conclusion, we have identified a peptide with the amino acid sequence HSSKLQ that can be used both as a substrate to measure PSA enzymatic activity in extracellular fluids and as a carrier to target prodrugs for activation within sites of metastatic prostate cancer producing enzymatically active PSA. We have documented that the PC-82 human prostatic cancer cells secrete enzymatically active PSA into their extracellular fluid. Thus, the PC-82 xenograft model can be used for testing whether therapeutic prodrug activation is possible *in vivo*. We have also demonstrated that PSA is enzymatically active in the extracellular fluid obtained from both normal prostate tissue and prostate cancer. We are continuing this work by coupling specific amino-TG analogues to the peptide HSSKLQ to evaluate their efficacy against PC-82 tumors *in vivo*.

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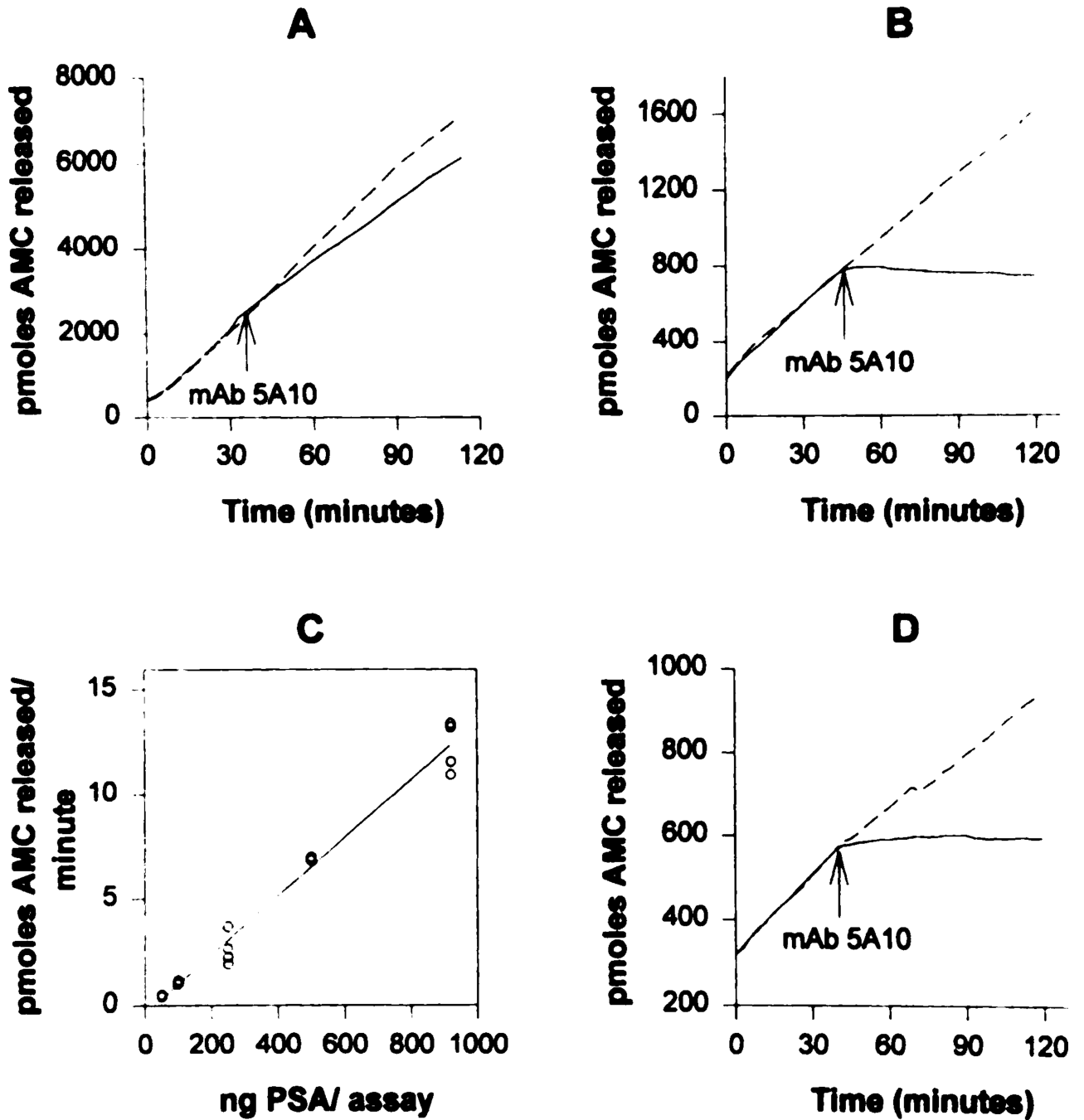


Fig. 1. Fluorometric assays using HSSKLQ-AMC substrate (0.4 mM) with neutralization of activity at indicated times by anti-PSA mAb 5A10 (10 µg/ml). *A*, PC-82 tumor homogenized in PSA assay buffer and assayed for PSA concentration by Tandem-R assay and aliquots containing 1 µg of total PSA assayed for activity. *B*, activity of PSA (1 µg) purified from human seminal plasma immunoprecipitated with mAb H117 (5 µg/ml) and protein A-Sepharose. *C*, linear range of enzymatic activity of immunoprecipitated PSA (human seminal plasma) using increasing amounts of PSA. *D*, activity of PSA (500 ng) immunoprecipitated from

PC-82 tumor homogenates., activity without mAb 5A10; —, activity with addition of mAb 5A10 at indicated times. All PSA assays were done in triplicate, and representative plots are shown.

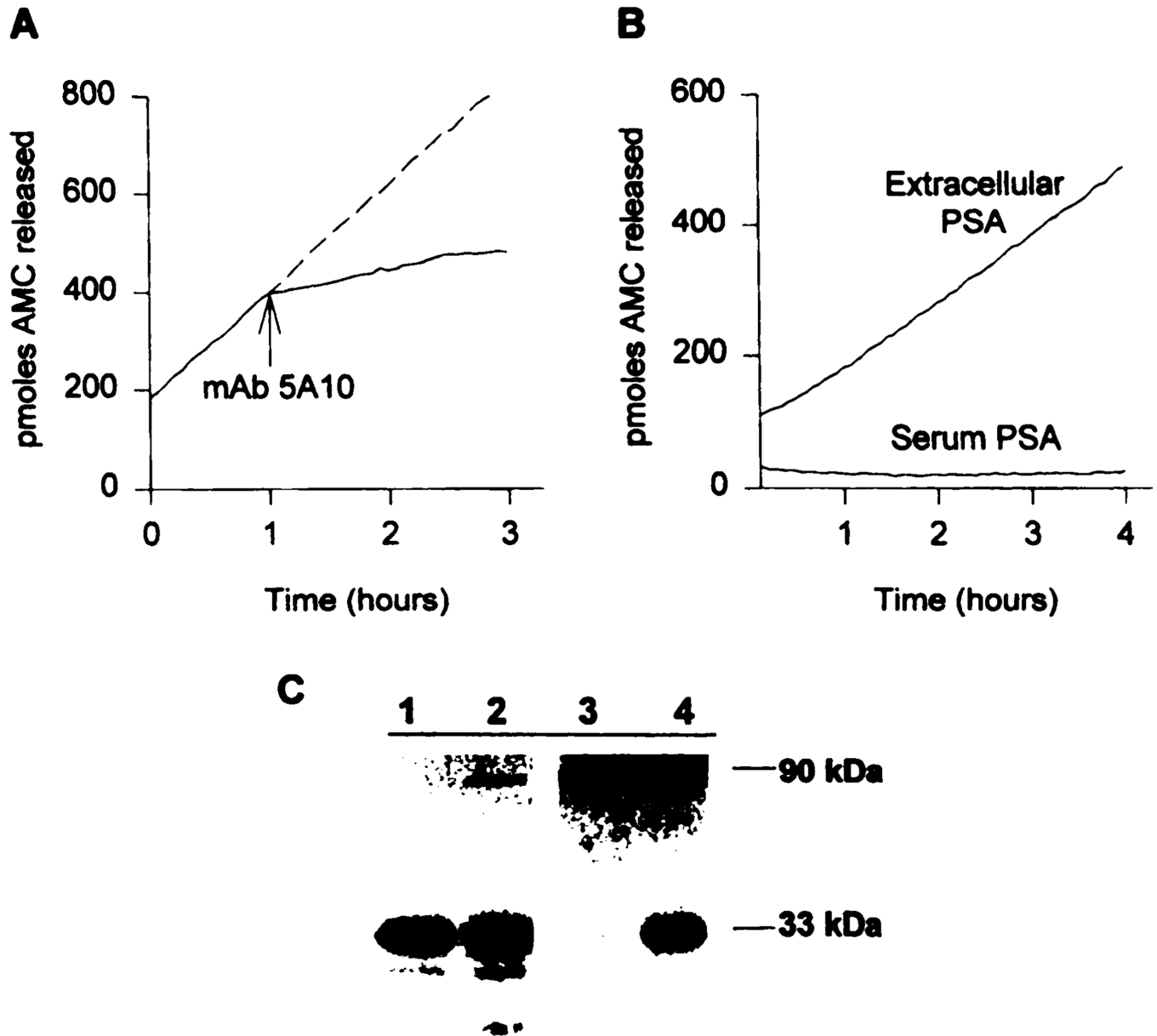


Fig. 2.

A, enzymatic activity of PSA (400 ng) immunoprecipitated from PC-82 tumor extracellular fluid using HSSKLQ-AMC substrate (0.4 mM) with and without the addition of mAb 5A10 (10 µg/ml) at indicated times. *B*, activity of PSA (200 ng) immunoprecipitated from pooled sera of PC-82 tumor-bearing animals compared to similar amounts of PSA immunoprecipitated from PC-82 tumor extracellular fluid. *C*, Western blot analysis of immunoprecipitated PSA (25 ng PSA/lane). *Lane 1*, purified human seminal plasma PSA; *Lane 2*, purified PSA incubated for 24 h with 10-fold molar excess ACT; *Lane 3*, PSA from pooled serum of PC-82 tumor-bearing animals; *Lane 4*, PSA from extracellular fluid from PC-82 tumor. All PSA assays were done in triplicate, and representative plots are shown.

Table 1

PSA substrate hydrolysis by purified extracellular proteases^d

Substrate group	Peptide sequence ^b	Pmol of substrate hydrolyzed/min/100 pmol of indicated protease									
		PSA	Chymotrypsin	Elastase	Trypsin	Urokinase	Plasmin	TPA	Thrombin	hK1	hK2
1	KGISSQY	510	4290	3068	658	UD	ND	ND	UD	ND	UD ^c
	SRKSQQY	234	3630	2788	260	0.4	0.4	UD	UD	2.0	UD ^c
	GQKGQHY	49.6	4274	922	122	ND	UD	0.6	UD	ND	UD ^c
2	EHSSKLQ	31.6	UD	UD	UD	UD	UD	UD	UD	UD	UD ^c
	QNKISYQ	16.8	9.0	9.0	UD	ND	UD	0.4	0.4	ND	UD ^c
	ENKISYQ	14.4	3.6	2.6	1.8	UD	0.6	0.4	0.3	UD	ND
3	ATKSKQH	16.0	38.8	UD	UD	UD	UD	UD	0.2	UD	ND
	KGLSSQC	13.6	12.2	950	UD	UD	UD	UD	UD	UD	ND
	LGGSQL	6.0	314	94.0	UD	UD	UD	UD	UD	ND	ND
4	QNKGHYQ	1.8	0.2	0.6	UD	UD	UD	UD	UD	UD	ND
	TEERQLH	1.2	48.8	1.0	UD	0.4	3.8	UD	1.0	UD	UD ^c
	GSFSIQH	UD	3.6	0.4	0.4	ND	0.2	UD	0.4	ND	ND
2	HSSKLQ	62.7	UD	UD	UD	UD	UD	UD	UD	UD	UD
	SKLQ	29.6	UD	UD	UD	UD	UD	ND	ND	UD	ND
	KLQ	UD	10.6	0.4	UD	ND	ND	ND	ND	UD	ND
	LQ	UD	UD	UD	UD	ND	UD	UD	UD	ND	ND
	Q	UD	UD	0.7	UD	UD	UD	ND	ND	ND	ND

^aTPA, tissue plasminogen activator; UD, undetectable (<0.1 pmol of substrate hydrolysis/min/100 pmol of protease); ND, not done; hK1, human plasma kallikrein; hK2, human glandular kallikrein.^bSubstrates at 0.2 mM concentration in PSA assay buffer [50 mM Tris-0.1 M NaCl (pH 7.8)], except LGGSQL, GSFSIQH, SKLQ, KLQ, and LQ, which were in 1.4% acetonitrile-PSA buffer and Q-AMC, which was in 0.2% formic acid-PSA buffer (pH 7.8).^chK2 tested at 17 pmol/assay except HSSKLQ-AMC at 100 pmol.

Table 2

Kinetic parameters of PSA peptide substrates

Group	PSA substrate ^a	K_m (μM)	k_{cat} (s^{-1})	$k_{\text{cat}}:K_m$ ($\text{s}^{-1} \text{M}^{-1}$)
1	KGISSQY	160	0.043	270
	SRKSQQY	90	0.023	260
2	EHSSKLQ	1165	0.012	10.6
	HSSKLQ	470	0.011	23.6
	SKLQ	813	0.020	24.6
	ATKSKQH	1310	0.0091	6.9
3	KGLSSQC	300	0.0017	5.6
	LGGSSQL	900	0.0037	4.1

^aPSA-mediated hydrolysis of substrates in buffer [50 mM Tris and 0.1 M NaCl (pH 7.8)].

Table 3

Stability of PSA substrates in various sera^a

Group	Sequence ^b	pmol of AMC released/min					
		Human ^c	Mouse	Rat	Fetal calf	Calf	Goat
1	SRKSQQY	UD	3.52	0.52	0.98	1.05	0.36
	GQKQHY	0.09	12.86	2.74	2.90	0.78	1.78
2	EHSSKLQ	UD	2.17	0.06	UD	UD	UD
	QNKISYQ	1.54	1.67	10.7	11.60	UD	5.54
	ENKISYQ	1.27	3.71	11.2	9.59	UD	3.18
3	ATKSKQH	UD	0.60	4.31	1.19	UD	0.35
	KGLSSQC	UD	0.66	0.80	0.03	UD	UD
	LGGSQL	UD	13.92	0.17	1.10	UD	0.11
4	QNKQHYQ	0.24	9.57	7.03	4.53	UD	3.47
	TEERQLH	UD	10.71	0.66	0.02	UD	UD
2	HSSKLQ	UD	0.81	1.04	0.31	UD	UD
	SKLQ	UD	1.70	UD	0.21	0.02	ND

^aUD, undetectable (<0.01 pmol/min change); ND, not done.^bEach substrate at 0.2 mM.^c100% sera used for each assay.

Table 4

PSA substrate hydrolysis by purified intracellular proteases^a

Peptide sequence ^b	PSA	pmol of substrate hydrolyzed/min/200 pmol of protease			
		B	C	D	Esterase ^c
EHSSKLQ	31.6	4.3	UD	2.2	UD
HSSKLQ	62.7	17.3	UD	3.8	UD
SKLQ	29.6	31.2	UD	6.4	UD
KLQ	0.4	87.0	UD	ND	UD
LQ	UD	190	UD	20.0	UD
Q	UD	1.0	UD	1.1	0.9

^aUD, undetectable (<0.1 pmol substrate of hydrolysis/min/200 pmol of protease); ND, Not done.

^bSubstrates at 0.2 mM concentration in PSA assay buffer except SKLQ, KLQ, and LQ, in 1.4% acetonitrile/buffer, and Q-AMC, in 0.2% formic acid/buffer (pH 7.8).

^cEsterase from porcine liver.