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Expression and Purification of Recombinant Active Prostate-Specific Antigen from *Escherichia coli*

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Abstract Human prostate-specific antigen (PSA), a 33 kDa serine protease with comprehensive homology to glandular kallikrein, is secreted from prostatic tissue into the seminal fluid and enters into the circulation. The level of PSA increases in the serum of patients with prostatic cancer and hence is widely employed as a marker of the disease status. In particular, an enzymatically active PSA that is a form cleaved at the N-terminal seven-amino-acids prosequence, APLILSR, of proPSA may play an important roll in the progression of prostate cancer. Thus, the presence of the active form would selectively discriminate the cancer from benign prostatic hyperplasia. In this study, we developed a convenient purification method for the acquisition of active PSA and proPSA. Recombinant proPSA and active PSA were expressed directly in *Escherichia coli*, easily and efficiently isolated from inclusion bodies, refolded, and purified. Moreover, the enzymatic activity of the recombinant active PSA was confirmed as serine protease using chromogenic chymotrypsin substrate. This purified active PSA could be further applied to scrutinize the biological or conformational characteristics of the protein and to develop specific diagnostic and/or therapeutic agents against prostate cancer.

Keywords: *Escherichia coli*, expression, prostate-specific antigen, purification, recombinant protein

Prostate cancer is one of the most common male malignant diseases and the second most leading cause of prostate-related death in North America [5]. Moreover, the number of prostate cancer patients is increasing significantly in the Asian countries including Korea. Although the early diagnosis of prostate cancer has been improved, cancer-specific diagnosis and therapeutic modalities are not yet completely developed. A human prostate-specific antigen (PSA) was first purified from prostatic tissues using gel electrophoresis [28], and the elevated serum levels of PSA was well correlated with the tumor volume and clinical stage of prostate cancer in a large group of patients [23]. Therefore, its level in the serum has been broadly used as an important marker for the diagnosis and follow-up of prostate cancer. However, usage of the serum level of PSA has been limited in the screening and early detection of prostate cancer because its level increases in men not only with prostate cancer but also with benign prostatic hyperplasia (BHP) or prostasis [6].

PSA belongs to the human glandular kallikrein family of serine proteases and is named human kallikrein 3 (hK3). The kallikrein (hK) gene family, located on chromosome 19q13.3-q13.4, consists of three members, hK1, hK2, and hK3 (PSA), and contains 15 genes [8, 15]. The hK1 pancreatic-renal kallikrein is produced mainly in the kidney, pancreas, and submandibular salivary glands. On the other hand, the hK2 and PSA are synthesized primarily in the prostate epithelium [8, 9]. PSA has nearly 70% amino acid sequence identity with hK1 and 80% with hK2 [9]. In addition to their amino acid sequence homology, hK2 and PSA share 78% amino acid structure homology with chymotrypsin-like substrate specificity [3, 27]. PSA is produced mainly by normal and malignant prostatic epithelial cells, secreted into seminal fluid, and enters into blood circulation.

PSA is primarily synthesized as preproPSA containing 261 amino acids with a leader sequence that contains a 17-amino-acids signal peptide located at its N-terminus. The preproPSA is cleaved at the signal peptide sequence, which cotranslationally generates an enzymatically inactive 244 amino acid precursor protein (termed proPSA) [1]. proPSA generates serine protease activity upon cleavage by trypsin or hK2 [26]. Specifically, hK2 functions as an *in vitro* activator and presumably also as a physiologic activator of proPSA [12]. This proPSA is cleaved at the N-terminal seven-amino-acids prosequence, APLILSR, by

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the activator [12, 26]. This activated proPSA is termed "active PSA." PSA exists in various molecular forms including the enzymatically highly active PSA, inactive proPSA, and nicked inactive PSA. In addition, PSA is mainly complexed with the protease inhibitor α-antichymotrypsn (ACT), whereas 5–35% of PSA is free in circulation [5]. The various forms of PSA could be used as diagnosis targets for prostate cancer. For example, the fraction of complexed PSA is higher than free PSA in the serum of prostate cancer patients, when compared with BPH patients [24]. In addition, PSA in BPH serum is present as a more extensively nicked inactive form than that in the prostate cancer serum [7]. PSA may be secreted into circulation as a more active state from prostate tumor tissues than from BPH tissue, which might explain the larger proportion of complexed PSA in the serum from prostate cancer [5]. Therefore, the measurement of the serum level of free active PSA that remains uncomplexed will be a specific approach to cancer diagnosis. The main biological function of PSA is to cleave the gel-forming proteins semenogelins 1 and 2 in semen. In addition, PSA was shown to expedite the growth of prostate metastases by increasing the bioavailability of insulin-like growth factor-1 (IGF-I) through promoting cleavage of insulin-like growth factor binding protein-3 (IGFBP-3) [17]. Noticeably, the active PSA could possibly play a role in the growth, invasion, and metastasis of prostate cancer cells *via* digestion of extracellular matrix components [25, 29]. Therefore, specific reagents that recognize various isoforms of PSA, especially including enzymatically active PSA, are of potential use in prostate cancer diagnostics and/or therapeutics.

Active PSA has been made mainly through difficult purification steps from human seminal fluid [11, 21, 31]. Alternatively, recombinant proPSA was expressed and purified using bacterial or mammalian expression systems, and then the active PSA was generated *via* enzymatic activation of the purified proPSA with trypsin or hK2 [4, 12, 26]. In this study, we established a much more convenient system to express recombinant proPSA and active PSA directly in *Escherichia coli* and a one-step strategy to purify the enzymatically active PSA without enzymatic digestion of the proPSA. This active PSA will be useful to study the structural and biological roles of the protein, and moreover, for the development of specific reagents to modulate prostate cancer.

MATERIALS AND METHODS

Cell Culture

The human prostate carcinoma cell line LNCaP was purchased from ATCC and maintained in RPMI 1640 medium (JBI) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10% fetal bovine serum. The cells should be allowed to incubate on undisturbed condition for the first 48 h after subculture. They were maintained at a density between 1×10^4 and 1×10^5 cells/cm^2 plate. Medium renewal was attended to twice per week.

Cloning of PSA cDNA

To obtain a PSA cDNA, total RNA was isolated from LNCaP cells by the acidic phenol-guanidinium thiocyanate-chloroform extraction method as previously described [14] and RT-PCR was performed with the RNA. Five μg of total RNA was incubated with 0.5 mM oligo(dT) primer at 65°C for 5 min, and the mixtures were cooled at room temperature for 10 min to obtain the primer-template annealing substrate. The annealed template was incubated with 5× first-strand buffer containing 1 mM dNTP and 30 U AMV reverse transcriptase (Promega) at 37°C for 30 min to obtain proPSA cDNA. The reaction was terminated by heating at 95°C for 5 min. The cDNA was then amplified by polymerase chain reaction as previously described [18]. Twenty ng of the cDNA was incubated with 25 pmol proPSA-specific primers (5' primer, 5'-CCGGAATTCGC-ACCCCTCATCCTGT-3'; 3' primer, 5'-CCCAAGCTTTC-AGGGGTTGGCCACGAT-3'), 10× reaction buffer, 2 mM dNTP, and 2.5 U pyrobest Taq DNA polymerase (Takara) in a 100-μl final volume and subjected to 30 rounds of temperature cycling at 95°C (30 sec), 55°C (30 sec), and 72°C (90 sec). The active PSA construct was generated by PCR amplification using specific primers from proPSA cDNA (5' primer, 5'-CCGGAATTCATTGTGGGAGGCTG-GGAG-3'; 3' primer, 5'-CCCAAGCTTTCAGGGGTTGG-CCACGAT-3').

Construction of Expression Vectors for proPSA and Active PSA

The proPSA cDNA was cleaved with EcoRI and HindIII for 3 h at 37°C, and cloned into pET28(a)+ vector using BL21 strain of *E. coli*. The sequence of the proPSA cDNA was confirmed by sequencing analysis. The recombinant vector was then cleaved with EcoRI and XhoI, and inserted into pGEX4T-1 expression vector. The expression vector for the active PSA was constructed by the same method as above.

Expression and Purification of Active PSA and proPSA

E. coli strain BL21(DE3) transformed with pGEX4T-1-proPSA or -active PSA was grown at 37°C in 2 l of ordinary LB medium with 0.1 mg/ml ampicillin. When the absorbance A_{600} had reached 0.6–1.0, isopropyl-β-D-thiogalactoside (Sigma) was added at 0.6 mM and incubated for further 5 h. The cells were then harvested and washed

with STE buffer (150 mM NaCl, 7.5 mM Tris-HCl, pH 8.0, 3 mM EDTA). The cells were stored frozen overnight at −80°C. The frozen cells were resuspended with STE buffer containing 1 mg/ml lysozyme, and placed for 15 min on ice. Five mM dithiothreitol (Sigma) and 1% N-lauryl sarcosyl (Sigma) were then added on ice for 5 min, and the mixtures were sonicated (50% power and 70% dulation with an ultrasonic disruption) ten times for 30 sec and chilled on ice. The shattered cells were spun off, and the supernatant was incubated with the preequilibrated 0.5 ml glutathione agarose beads (glutathione Sepharose 4B, Amersham Biosciences) by rotating at 4°C overnight. The sample was then flowed through a polypropylene column (Qiagen) and washed with 100 ml STE buffer. Recombinant proPSA or active PSA protein was acquired by elution buffer (STE buffer containing 30 mM glutathione). The presence of the recombinant proteins was confirmed by their migration on SDS-polyacrylamide gel electrophoresis and detection using protein staining solution (45% methanol, 10% glacial acetic acid, 0.025% Coomassie brilliant blue R-250).

Refolding and Concentration of the Active PSA and proPSA

The eluted proteins were refolded by dialyzing with membrane (spectra/PorR Membrane, MWCO:3500, Spectrum Laboratories Inc.) in 3 l of refolding buffer (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, 2 mM potassium phosphate) and by slowly stirring for 24 h at 4°C. The refolding buffer was changed 2 times per 12 h. The refolded proteins were concentrated using centricon (YM-30, Millipore), and the final protein concentration was ~200 ng/ml.

Western Blot Analysis

The purified proteins were subjected to electrophoresis on 10% (w/v) denaturing SDS-polyacrylamide gels and then transferred electrophoretically onto a PVDF membrane as previously described [19]. Nonspecific protein binding was blocked by incubation with 5% skim milk (DIFCO) and 0.1% Tween 20 (USB) in PBS at room temperature for 90 min. The membrane was then incubated with rabbit polyclonal anti-PSA primary antibody (Biomeda) in PBS containing 0.1% Tween 20 at 4°C overnight. After five washes with PBS with 0.02% Tween 20, membrane-bound antibody was detected with HRP-conjugated goat anti-rabbit IgG (Santa Cruz). The immune complexes were visualized by the enhanced chemiluminescence detection system.

Measurement of Enzymatic Activity of PSA

The purified proPSA or active PSA (200 pmol) was incubated at room temperature with 0.4 mM fluorogenic substrate, Mu-His-Ser-Ser-Lys-Leu-Gln-AFC (Calbiochem), in a final volume of 200 µl of the assay buffer containing 50 mM Tris-HCl (pH 7.9) and 100 mM NaCl, as previously described [10]. The hydrolysis of the substrate was monitored with the fluorospectrometer (Jasco), every 10 min for 90 min, using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. As a blank, the substrate alone in the reaction buffer was used. The observed fluorescence of each sample was normalized by subtraction with the background value from intrinsic fluorescence of the substrate itself. The amount of released AFC was then assessed based on the comparison with a standard curve of the fluorescence with a known concentration of AFC.

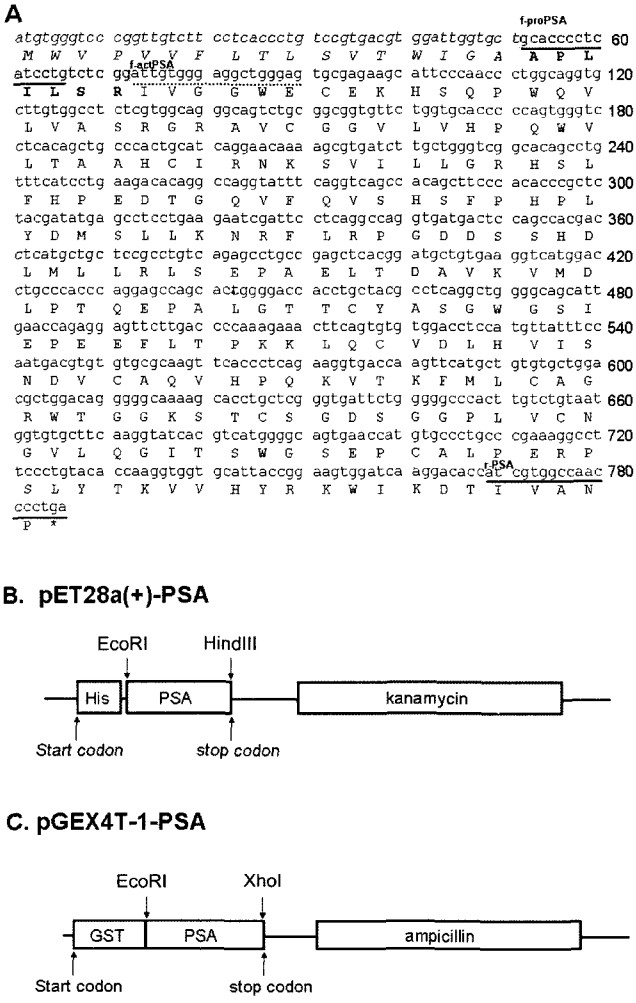

Fig. 1. Complete nucleotide sequence of preproPSA and schematic representation of expression constructs of PSA.

The nucleotide and deduced amino acid sequences of the X05332 cDNA (Gene Bank) are represented (**A**). Underline represents primer sites used for obtaining PSA cDNA. f-proPSA and r-PSA were primer sets for proPSA cDNA, and f-actPSA (dotted line) and r-PSA were primer sets for active PSA cDNA. The gray box denotes the prosequence that is cleaved for active PSA. The sequence forward to the prosequence (italicized sequence) represents the signal peptide sequence. The asterisk denotes the stop codon. Expression vectors for the recombinant proteins of active PSA and proPSA were constructed using his-tag (**B**) or GST-tagged (**C**) expression vectors.

RESULTS AND DISCUSSION

Cloning and Expression of proPSA and Active PSA

The sequences of the complete nucleotide and amino acids of the preproPSA and structure of the PSA expression vector are shown in Fig. 1. To obtain two different kinds of PSA cDNAs, proPSA and active PSA cDNA, total RNA of LNCaP cell was purified and amplified using RT-PCR with specific primers. The proPSA cDNA was primarily obtained with a 735-base-pair length, in which the signal peptide sequence was deleted (Fig. 1A and data not shown). Active PSA cDNA was then generated from the proPSA cDNA with RT-PCR. This active PSA cDNA had 714 base pairs in which 21 nucleotides of prosequence were removed from the proPSA cDNA (Fig. 1A and data not shown). Expression vectors for two forms of PSA, proPSA and active PSA, were then constructed using two different kinds of protein expression vectors. One is the vector expressing fusion protein tagged with six histidines [pET28a(+)]; the other is with glutathione-S-transferase protein (GST) (pGEX4T-1), which was tagged at the N-terminus of the PSA proteins (Figs. 1B and 1C). The identity of each cloned expression vector was confirmed through sequencing with the ABI PRISM 310 genetic analyzer.

Purification of proPSA and Active PSA

E. coli strain BL21 (DE3) was transformed with four different kinds of expression plasmids [pET28a(+)-proPSA, pET28a(+)-active PSA, pGEX4T-1-proPSA, pGEX4T-1-active PSA]. The cells with each vector were cultured and harvested 5 h after induction at 37°C with 0.6 mM isopropyl-β-D-thiogalactoside. The collected cells were treated with lysozyme, sonication, and centrifugation, and then the majority of induced PSA proteins was found in the inclusion bodies (lanes 4 and 5 of Figs. 2A and 2B). Therefore, we tried to solubilize the inclusion bodies to purifiy the recombinant PSA proteins.

For the purification of his-tagged PSA proteins, cell extracts were treated with 1.5% N-Lauryl sarcosyl, 1% Triton X-100, 0.5% NP40, 6 M Gu-HCl, or 1 to 8 M urea. The treatment of more than 5 M urea was found to increase the solubility of the proteins into supernatant from inclusion bodies (data not shown). To remove the high concentration of the urea in the protein samples, we employed dialysis before binding of the proteins into nickel-chelate resin (Qiagen). However, the purified active PSA did not harbor protease activity, probably because both lengthy purification steps and urea treatment might prohibit the recovery of misfolded proteins (data not shown). As an alternative system for the PSA expression, we used GST-fusion expression vectors because the fusion of a protein to a large-affinity tag such as GST has been known to be advantageous in terms of increased expression, enhanced solubility, and improved folding [22]. For the

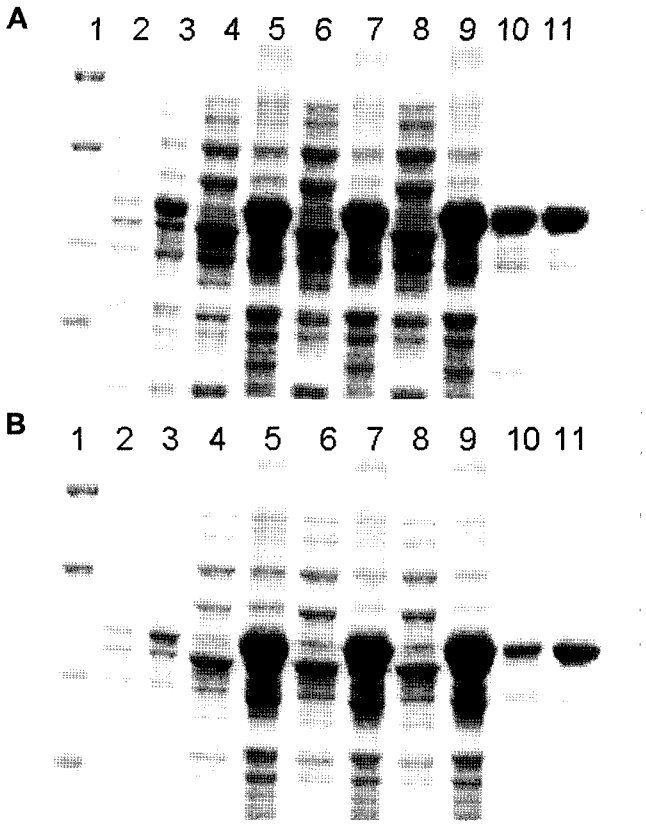

Fig. 2. The quantity of protein samples during different conditions for the purification of GST-fused proPSA (**A**) and active PSA (**B**). Lane 1, molecular mass markers (166 kDa, 66 kDa, 45 kDa, 35 kDa, respectively); lane 2, total cell extracts without induction; lane 3, total extracts after 5 h induction; lane 4, supernatant of extracted samples after induction; lane 5, pellet of extracted samples after induction. Proteins were extracted after addition of 1% IGEPAL CA-630 (lanes 6 and 7), 1% Triton X-100 (lanes 8 and 9), or 1% sarcosyl (lanes 10 and 11), and the supernatant (lanes 6, 8, and 10) or pellet samples (lanes 7, 9, and 11) were subjected to a 10% SDS-PAGE.

purification of GST-fused PSA proteins, cell extracts were treated with different kinds of mild detergent such as 1% IGEPAL CA-630, 1% Triton X-100, or 1% sarcosyl. The quantity of solubilized protein samples from inclusion bodies in each of the different conditions was monitored by 10% SDS-PAGE (Figs. 2A and 2B). The treatment of sarcosyl was found as the best condition for the solubility of the recombinant proteins (compare lanes 6, 8, and 10 of Figs. 2A and 2B).

Supernatant from sarcosyl-treated cell extracts was loaded onto glutathione agarose beads, and the proteins bound to the beads were eluted with glutathione (Figs. 3A and 3B). The eluted proteins were, however, found to be negligible protease activity (data not shown). Thus, the proteins were refolded by dialyzing, concentrated, and subjected to 10% SDS-PAGE. We then could observe a single band of proteins with expected molecular mass of approximately 55 kDa and 54 kDa from samples containing

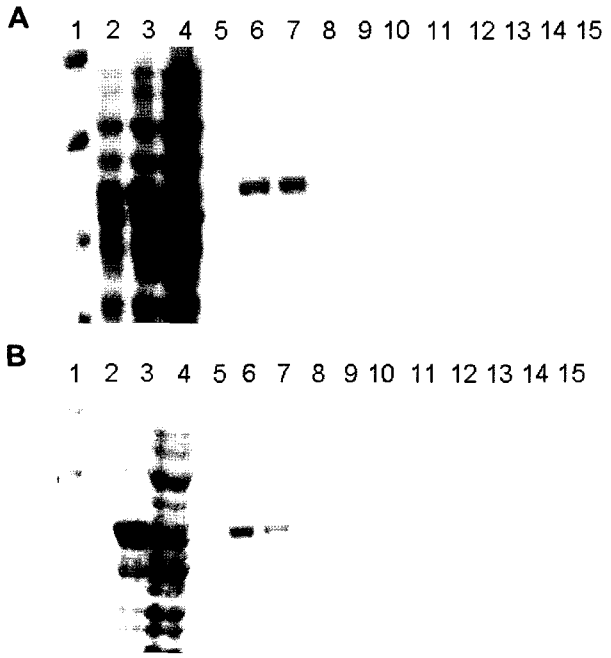

Fig. 3. SDS-PAGE analysis of GST-fused proPSA (**A**) and active PSA (**B**).
Lane 1, molecular mass markers (166 kDa, 66 kDa, 45 kDa, 35 kDa, respectively); lane 2, total cell extracts without induction; lane 3, total extracts after 5 h induction; lane 4, flow, through samples; lane 5, samples after last washing; lane 6, samples on a bead after washing; lane 7, samples on a bead after elution with glutathione; lanes 8–15, eluted samples. Proteins were analyzed using a 10% SDS-PAGE.

recombinant proPSA and active PSA, respectively (Fig. 4A). The identity of the protein band seen on the gels was confirmed by Western blotting analysis using an anti-PSA polyclonal antibody (Fig. 4B). In contrast, no PSA-specific proteins were detected from innate *E. coli* extracts used for the PSA purification in this study. These results strongly indicated that we had developed a simple and highly effective purification step for the recombinant proPSA and active PSA proteins from bacteria.

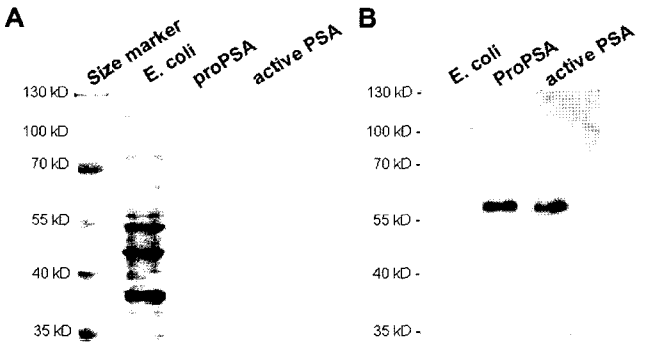

Fig. 4. SDS-PAGE (**A**) and Western blotting analysis (**B**) of the purified recombinant PSA proteins expressed in *E. coli*.
No specific bands were detected from extracts from the innate bacteria when reacted with PSA-specific polyclonal antibody.

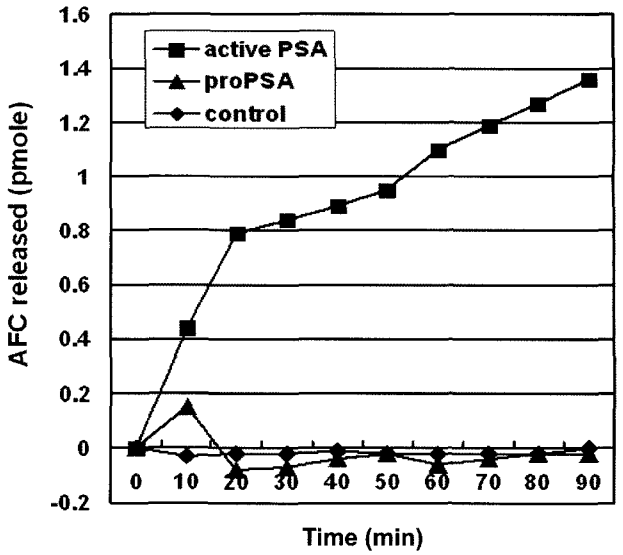

Fig. 5. Fluorometric assay for PSA enzyme activity using Mu-His-Ser-Ser-Lys-Leu-Gln-AFC substrate.
Recombinant PSA proteins (200 pmoles) were mixed with the fluorogenic substrate (0.4 mM) dissolved in assay buffer. Increase in fluorescence was detected for 90 min, and the moles of released AFC were calculated using a standard curve with a known quantity of AFC.

Enzymatic Activity of Active PSA

To confirm that the purified recombinant active PSA contains serine protease activity, a highly specific fluorogenic substrate for the PSA was used to assess the enzymatic activity (Fig. 5). The substrate used in this study had great specificity for PSA, and fluorescent peptide can be generated by the protease treatment [10]. Results showed that the purified proPSA did not contain protease activity with comparable basal level in the presence of substrate only. By large contrast, the recombinant active PSA generated efficient fluorescence when reacted with the substrate. Moreover, the amount of fluorescence was increased with dependency on the incubation time. From the standard experiment of fluorescence observation with increasing known amounts of AFC, the specific activity of the purified PSA from this study was 0.004 U/mg (1 unit defines the ability of PSA to release 1 nmole of AFC from the substrate per min at 25°C). Conclusively, the purified GST-fused active PSA contained enzymatic activity as serine protease. Although active PSA protein was expressed and obtained from *E. coli.* as a GST-fused recombinant form, this result suggested that the purified protein harbored nearly the same conformation as natural active PSA protein existing in the blood of humans.

The purification strategy of PSA using other systems such as mammalian cells or insect cells required also refolding of the protein from inclusion bodies and/or multiple column chromatography steps, both resulting in fairly low yields of purified protein [2, 13, 28]. In this study, we established a convenient expression system and

easy purification step in *E. coli* for the acquisition of active PSA. The obtained active PSA through this system had enzymatic activity as serine protease. Thus, we could produce native PSA proteins with speed and ease using the methods developed in this study. Recently, enzymatically active and inactive PSA were reported to be selectively separated or recognized by PSA-binding peptide or anti-PSA monoclonal antibodies [16, 30]. The active PSA purified in this study can be further used not only to study the biological or structural functions of the protein but also to select specific ligands such as the peptide or RNA aptamer [20] for detection and/or therapy of prostate cancer.

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