

Characterization of recombinant human protein C inhibitor expressed in *Escherichia coli*

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Abstract

The serine protease inhibitor (serpin) protein C inhibitor (PCI; also named plasminogen activator inhibitor-3) regulates serine proteases in hemostasis, fibrinolysis, and reproduction. The biochemical activity of PCI is not fully defined partly due to the lack of a convenient expression system for active rPCI. Using pET-15b plasmid, Ni²⁺-chelate and heparin-Sepharose affinity chromatography steps, we describe here the expression, purification and characterization of wild-type recombinant (wt-rPCI) and two inactive mutants, R354A (P1 residue) and T341R (P14 residue), expressed in *Escherichia coli*. Wild-type rPCI, but not the two mutants, formed a stable bimolecular complex with thrombin, activated protein C and urokinase. In the absence of heparin, wt-rPCI-thrombin, -activated protein C, and -urokinase inhibition rates were 56.7, 3.4, and $2.3 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$, respectively, and the inhibition rates were accelerated 25-, 71-, and 265-fold in the presence of 10 $\mu\text{g/mL}$ heparin for each respective inhibition reaction. The stoichiometry of inhibition (SI) for wt-rPCI-thrombin was 2.0, which is comparable to plasma-derived PCI. The present report describes for the first time the expression and characterization of recombinant PCI in a bacterial expression system and demonstrates the feasibility of using this system to obtain adequate amounts of biologically active rPCI for future structure–function studies.

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1. Introduction

Protein C inhibitor (PCI; systematic name *SERPINA5*; also named plasminogen activator inhibitor-3) is a 57 kD glycoprotein that inhibits numerous serine proteases includ-

ing activated protein C, thrombin (free and bound to thrombomodulin), urokinase, acrosin, and plasma kallikrein [1–10]. The physiological function of PCI in blood coagulation and fibrinolysis has been widely studied but its physiological relevance is not fully understood in part due to its apparent dual anti- and pro-coagulant activities [11–15]. Interestingly, PCI is also critically involved in reproduction since male homozygous PCI knock-out mice are infertile and show impaired spermatogenesis [16]. In humans, seminal plasma is a major source of PCI where it acts as the inhibitor of acrosin [17], human kallikrein 2 [18] and the plasminogen activators, urokinase and tissue plasminogen activator [5]. PCI would locally prevent non-appropriate proteolytic activities that could interfere with normal reproduction mechanism. Recently, PCI has been studied in cancer biology using PCI-transfected MDA-MB-

Abbreviations: APC, activated protein C; IPTG, isopropyl-beta-D-thiogalactopyranoside; LB medium, L-broth medium; Ni-NTA, nickel-nitrilotriacetic acid; rPCI, recombinant protein C inhibitor; P1-rPCI, R355A rPCI; P14-rPCI, T341R rPCI

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435 breast cancer cells and PCI increased both adhesion and motility to vitronectin and fibronectin [19]. Protein C inhibitor has also been identified in malignant prostate, which suggests a potential role for PCI in cancer progression [20].

Protein C inhibitor belongs to the *serine protease inhibitor* superfamily (serpin), which contains more than 400 members [21–23]. Serpins share the same overall structure, composed of 3 β sheets and 9 α helices [21,22]. Their inhibitory activity follows an original scheme where the target protease interacts with the reactive site loop of the serpin and cleaves it at the reactive site P1–P1' (according to the Schechter and Berger nomenclature [24]). This interaction leads either to the formation of a covalent complex between the protease and the serpin where both proteins become inactive (the inhibitory pathway) or to the generation of an inactive irreversible clipped form of the serpin while the protease recovers its activity (the substrate pathway). PCI interaction with proteases is characterized predominantly by the formation of a covalent complex, although some clipped forms of PCI have been identified in biological fluids as well as in *in vitro* experiments [25]. Besides its ability to regulate protease activities, PCI also binds heparin [26–28] and retinoic acid [29]. Many PCI–protease inhibition reactions are increased by heparin [26–28]. The binding of retinoic acid to PCI has no effect on its inhibitory activity but this interaction may have a role in hormone regulation [29]. The three-dimensional structure of clipped PCI was recently described, and Huntington et al. proposed independent binding sites for heparin and retinoic acid [30].

Having a convenient recombinant PCI expression system allowing high-level expression of PCI and the production of PCI mutants would provide a new tool for further structure/function studies. A recombinant PCI was obtained using baculovirus and insect cells [31–34]. There have been other rPCI expression systems described including baby hamster kidney cells [35] and human embryonic kidney cells [36]. However, insect cell expression and other eukaryotic systems can be somewhat challenging and time-consuming, while a bacterial expression system provides an easy alternative for rapid protein production and at a lower cost. This work reports the expression of wild-type recombinant PCI (wt-rPCI) using an *Escherichia coli* system. By site-directed mutagenesis, we also prepared two inactive rPCI mutants, R354A-rPCI (P1-rPCI) and T341R-rPCI (P14-rPCI). We assessed the activity of wt-rPCI and the non-inhibitory mutants with thrombin, activated protein C, and urokinase in the absence and the presence of heparin.

2. Materials and methods

2.1. Materials

Human α -thrombin and urokinase were respectively purchased from Haematologic Technologies Inc. (Essex

Jct., VT, USA) and American Diagnostica (Greenwich, CT, USA). Human wild-type recombinant activated protein C (APC) was a gift from Dr. Brian W. Grinnell (Lilly Research Laboratories, Indianapolis, IN, USA). Human plasma PCI was from Affinity Biological Inc. (Ontario, Canada). Tos-Gly-Pro-Arg-pNA and Pefachrome PCa were obtained from Pentapharm (Basel, Switzerland) and S-2444 substrate was from Chromogenix (Milano, Italy). Heparin was from Diosynth Inc. (Oss, the Netherlands). Imidazole and polybrene were from Sigma-Aldrich Co. (St Louis, MO, USA); lysozyme from Worthington Biochemical Co. (Freehold, NJ, USA). PolyHis (0.2 $\mu\text{g}/\mu\text{l}$, Novagen, Madison, WI, USA) and human PCI (0.1 $\mu\text{g}/\mu\text{l}$) monoclonal antibodies were diluted in non-fat milk. Anti-mouse peroxidase conjugate antibody (1/15,000 in non-fat milk) was from Sigma-Aldrich Co. (St Louis, MO, USA).

2.2. Cloning and mutagenesis of PCI

The cDNA for human PCI was cloned between the *Nde*I and *Bam*HI restriction sites in the pET-15b plasmid (Novagen). This vector includes a 6xHis tag followed by a sequence for further thrombin cleavage at the 5' terminal of the polylinker. Point mutations were introduced according to the QuickChange Site-directed Mutagenesis Kit procedure (Stratagene), using the following primers to generate P1 and P14 mutants respectively: 5'-GCG GCA GCC ACG GGG ACA ATC ATC TTC ACT TTC GCG TCG GCC CGC CTG-3' (forward R354A), 5'-CAG GCG GGC CGA CGC GAA AGT GAA GAT TGT CCC CGT GGC TGC CGC-3' (reverse R354A) to 5'-GCT GTG GTG GAG GTG GAC GAG TCG GGA CGG AGA GCA GCG GCA GCC-3' (forward T341R), 5'-GGC TGC CGC TGC TCT GCG TCC CGA CTC GTC CAC CTC CAC CAC AGC-3' (reverse T341R). Supercompetent XL1-Blue cells were transformed by the heat shock method, according to the manufacturer's instructions, the positive clones were selected with ampicillin, and the plasmids were then purified and sequenced by the University of North Carolina Sequencing Core Facility to verify the incorporation of the mutations.

2.3. Expression and purification of recombinant PCI

Recombinant PCI in pET-15b was expressed in *E. coli* BL21(DE3)plyS grown at 30 °C in 2 L of LB with 50 $\mu\text{g}/\text{ml}$ carbenicillin and 34 $\mu\text{g}/\text{ml}$ chloramphenicol. At an OD₆₀₀ of 0.6–0.8, IPTG (isopropyl-beta-D-thiogalactopyranoside) was added to a final concentration of 1 mM. Five hours after the addition of IPTG, cells were collected by centrifugation at 4000 \times g for 20 min and frozen at –80 °C. Recombinant PCI was purified under native conditions according to the Qiagen procedure for purification of His-tagged proteins. Cells were thawed for 1 h on ice, brought to a final volume of 40 ml with lysis buffer containing 40 mg lysozyme and incubated on ice for an hour. Cells were

disrupted by sonication on ice with six 10-s bursts and 10 s pauses. Cell debris was pelleted at $9500\times g$ for 30 min and the supernatant mixed with 5 ml of NiAgarose beads (nickel-nitrilotriacetic acid agarose, Qiagen Inc., Valencia, CA) and rocked at 4 °C for 30–60 min. The NiAgarose beads were loaded onto a column and washed with 10 mM imidazole in 300 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0 (NaPi). Bound proteins were then eluted with 50 mM imidazole in NaPi followed by 250 mM imidazole in NaPi. EDTA was added to the eluted pooled fractions at a final concentration of 10 mM. The eluted proteins were diluted 3-fold in HNPE (20 mM Hepes, 130 mM NaCl, 0.1% polyethylene glycol buffer, 0.02% NaN_3 , 10 mM EDTA, pH 7.4), and loaded onto a 1 ml HiTrap heparin column (Amersham Biosciences) equilibrated in HNPE. The column was washed with 10 ml HNPE, and bound proteins eluted with HNPE containing 0.5 M NaCl, followed by elution with HNPE containing 2 M NaCl (rPCI may elute at lower ionic strengths depending upon the source of immobilized-heparin). Pooled fractions of rPCI were dialyzed into an HNPE buffer. Dialyzed rPCI was centrifuged at $12,000\times g$, at 4 °C for 20 min. Concentration was calculated by A_{280} , $E_{280\text{ nm}}=1\text{ (mg/ml)}^{-1}$ or $E_{280\text{ nm}}=0.048\text{ (\mu M)}^{-1}$ using 45.9 kD as the M_r of the polyHis-tagged rPCI. The purity of the PCI protein was analyzed by SDS-PAGE under denaturing conditions.

2.4. Gel analysis of PCI and its mutants upon interaction with thrombin

Interaction of wt-rPCI and non-inhibitory mutants with thrombin was analyzed by SDS-PAGE. Thrombin (1 μM) and PCI (1 μM) were incubated in 20 mM Hepes, 130 mM NaCl, 0.2% polyethylene glycol 8000, and 0.02% NaN_3 , pH 7.4 (HNP buffer) for 1 h at 37 °C, and the reaction was stopped by boiling, and visualized by Coomassie blue staining. For immunoblot analysis, thrombin and PCI at equimolar concentrations (200 nM) were incubated as described above, submitted to SDS-PAGE and transferred onto a PVDF membrane that was blocked in Tris-buffered saline (TBS) containing 10% non-fat milk and 0.2% Tween 20 (v/v) at room temperature for 1 h, incubated with the primary antibody (monoclonal His-tag or monoclonal PCI antibodies) for 1 h at 37 °C, and washed in TBS prior to the addition of the secondary anti-mouse peroxidase conjugate antibody. The signal was detected using ECL reagents.

2.5. PCI protease inhibition assays

All assays were performed at room temperature in 96-well microtiter plates coated with 2 mg/mL BSA as described elsewhere [32–34]. In the absence of heparin, thrombin (1 nM) was incubated with 100 nM PCI in HNP buffer, pH 7.4, and 2 mg/ml BSA with 100 $\mu\text{g/ml}$ Polybrene (Sigma). At timed intervals, 150 μM tosyl-Gly-Pro-Arg-*p*-

nitroanilide was added in HNP buffer containing 100 $\mu\text{g/ml}$ Polybrene and substrate cleavage was measured at 405 nm using a plate reader (Molecular Devices). Inhibition assays with rPCI and urokinase (2 nM) [with S-2444 (1 mM)] and APC (1 nM) [with Spectrozyme PCa (150 μM)] were similarly performed except with these buffers: 50 mM Tris-HCl, 100 mM NaCl, pH 7.8 for urokinase and HNP containing 2.5 mM CaCl_2 for APC.

In the presence of heparin, 10 nM PCI was incubated with 1 nM thrombin, 0.01–2000 $\mu\text{g/ml}$ heparin and in the HEPES-buffer plus 2 mg/ml BSA. At timed intervals tosyl-Gly-Pro-Arg-*p*-nitroanilide (150 μM) was added in HNP buffer containing 2000 $\mu\text{g/ml}$ Polybrene. The reaction was stopped by adding 50 μl 50% acetic acid, the plates were centrifuged at $2000\times g$ for 20 min to remove the Polybrene-heparin precipitate, and the absorbance at 405 nm was measured in a microplate reader. Second order rate constants represent the average of at least three to five independent experiments done in triplicates [32–34]. A similar assay was used for urokinase (2 nM) and APC (1 nM) with a 10-fold molar excess of wild-type PCI in the presence of 0.01–2000 $\mu\text{g/ml}$ of heparin, except with these buffers: 50 mM Tris-HCl, 100 mM NaCl, pH 7.8 for urokinase and HNP containing 2.5 mM CaCl_2 for APC.

Stoichiometry of inhibition (SI value) was measured by incubating rPCI (2.5–80 nM) with active site titrated thrombin (10 nM) overnight at room temperature in HNP buffer at pH 7.4 plus 0.1 mg/ml ovalbumin. Residual thrombin activity was measured as described above and the data plotted as percentage thrombin activity remaining on the *y*-axis, and rPCI concentration plotted on the *x*-axis, and the *x*-intercept was used to calculate the SI value.

3. Results

3.1. Cloning and mutagenesis of wild-type human PCI, R354A PCI (P1-PCI) and T341R PCI (P14-PCI) variants

The human PCI cDNA was cloned in the pET-15b vector with an amino-terminal-linked 6-histidine sequence to facilitate the purification procedure using a nickel column, and this construct was transformed into the BL21(DE3)pLysS *E. coli* strain for protein expression (Fig. 1). Two independent mutations of PCI occurring at positions P1 and P14 of the reactive site loop were introduced in the PCI cDNA by site-directed mutagenesis (Fig. 1). Both mutations should abolish the inhibitory ability of PCI. We expect P1-rPCI R354A to lack inhibition activity for a serine protease with “trypsin-like” specificity. Previous studies reported that when Thr at the P14 position of the reactive site loop is mutated to Arg, for PCI this is T341R, the resulting serpin behaves as a substrate for protease, not an inhibitor [37,38]; thus, it is cleaved upon recognition by the protease but fails to trap the protease into a covalent complex.

His-Tag
 MGSSHHHHHHSSGLVPR↓GSHMHRHHPREMKKRVEDLHVGATVAPSSRRDFT
 FDYRALASAAPSQNIFFSPVSISMSLAML^HSLGAGSSTKMQILEGLGLNLQKS
 SEKELHRGFQQLLQELNQP^HRDGFQLSLGNALFTDLVV^HDLQDTFVSAMKTLYL
 ADTFPTNFRDSAGAMKQINDYVAKQTKGKIVDLLKNLDSNAV^HVIMVNYIFFK
 AKWETSFNHKGTQE^HQDFYVTSETVVRVP^HMMSREDQYHYLLDRNLS^HCRVVGVP
 YQGNATALFILPSE^HGKMQQVENGLSEKTLRKWLKMF^HKKRQLELYLPKFSIEG
 SYQLEKVLPSL^HGISNVFTSHADLSGISNHSNIQVSEM^HVHKAVVEVDES^HG^TRA
 AAATGTIFTF^R↓SARLNSQRLVFNRPF^RLMFIVDNNILFLGK^RVNRP

Fig. 1. Protein sequence of His-tagged human rPCI. ↓, thrombin cleavage site; ^H, residue+1 of human PCI sequence; ^T, P14 position; ^R, P1 position; ↓, reactive site (according to Schechter and Berger's nomenclature [24]).

3.2. Expression and purification of recombinant PCI molecules

The initial attempts to purify the wt-rPCI using a 6-h induction of protein expression with IPTG at 37 °C and a 250 mM imidazole elution resulted in the co-elution of rPCI with major impurities of 60 kD. Several modifications to the purification protocol were conducted, including the addition of the reducing agent dithreitol or mild detergent (cyclodextrin) to the wash buffer, or the use of denaturing conditions with 2.5 M urea, and the increase of the salt concentration from 1 M to 2 M in the wash and elution buffers. None of these adjustments changed the level of purity of rPCI fractions in the conditions used. The 60 kD proteins were identified as chaperone bacterial GroEL proteins by proteomics and mass spectrometry (data not included).

The improvement of the purification protocol was achieved by carrying out protein induction at 30 °C instead of 37 °C and adding 10 mM EDTA to the eluate to bind any free Ni²⁺ leaching off the column (Fig. 2). By diluting the eluate from the Ni²⁺-chelate column into the HNP buffer with EDTA, protein aggregates were also dramatically decreased. Additional purification was achieved with HiTrap heparin-Sepharose, which removed any denatured PCI molecules and other contaminants that co-purified on the Ni²⁺-chelate column (Fig. 2). Alternatively, rPCI could be purified by performing a two-step elution with 150 mM imidazole after the 50 mM imidazole wash, which eliminates some rPCI and most of the GroEL proteins, followed by 250 mM imidazole elution that contained highly purified rPCI; yet with an overall net loss of rPCI compared to using HiTrap heparin-Sepharose (Fig. 2). The

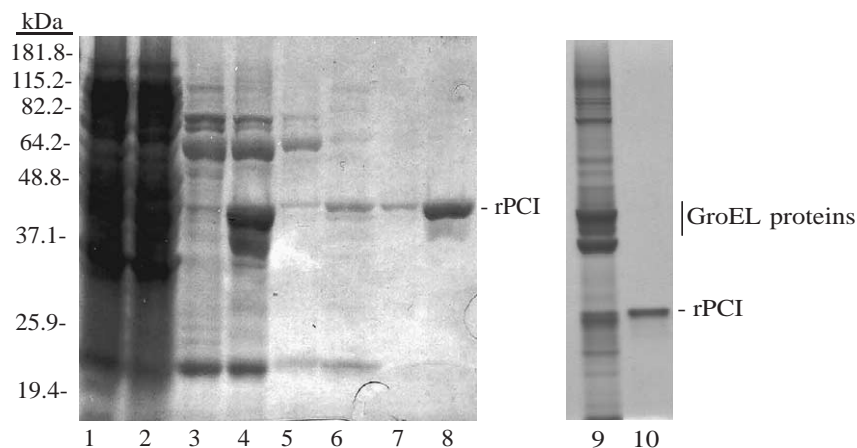


Fig. 2. SDS-PAGE analysis of His-tagged human rPCI expressed in *E. coli* and purified under native conditions using the Ni²⁺-chelate and heparin-Sepharose columns (left panel) or by a two-step imidazole elution using only the Ni²⁺-chelate column (right panel). 10 μL of each fraction was analyzed under non-reducing conditions and proteins were visualized using Coomassie blue staining. (Left panel) Lane 1, lysate; lane 2, Ni²⁺-chelate column flow-through; lane 3, 50 mM imidazole wash; lane 4, 250 mM imidazole elution; lane 5, HiTrap heparin-Sepharose flow through, lane 6, 0.5 M NaCl wash; lane 7, 2.0 M elution of rPCI with a concentration of 3 μM; lane 8, 2.0 M elution of rPCI with a concentration of 23 μM; (Right panel) lane 9, 150 mM imidazole elution (shown here are the major impurities that migrated at 60 kD identified as GroEL proteins); lane 10, 250 mM imidazole elution of rPCI (that migrated at the predicted M_r of rPCI) with a concentration of 2.5 μM.

rPCI mutants (P1 and P14) were also purified according the same protocols developed for wild-type rPCI. No major differences could be seen in the level of expression or purity of the proteins.

3.3. SDS-PAGE analysis of thrombin interaction with wt-rPCI, P1-rPCI and P14-rPCI

Thrombin and rPCI (wt and the two mutants) were incubated at equimolar concentrations for 1 h at 37 °C and the protein–protein interaction was assessed by SDS-PAGE. As visualized by Coomassie blue staining, all three rPCI

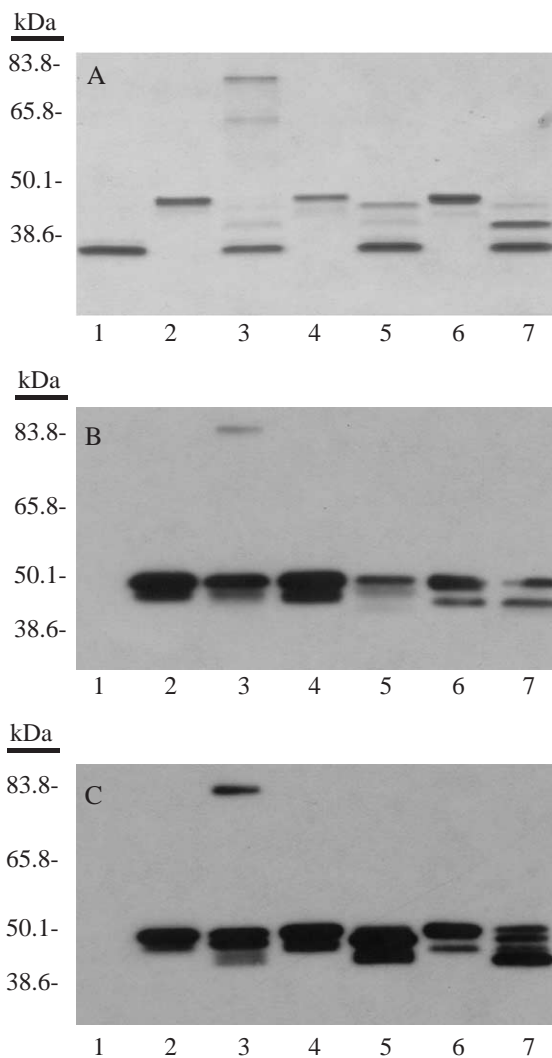


Fig. 3. SDS-PAGE analysis of the interaction between purified wild-type rPCI, P1-rPCI or P14-rPCI and thrombin. Thrombin and rPCI molecules were incubated for 1 h at 37 °C as detailed in Materials and methods and proteins were analyzed by (A) Coomassie blue staining, (B) immunoblot with a His-tag monoclonal antibody, or (C) immunoblot with a PCI monoclonal antibody. Results are representative of two independent assays. Lane 1, thrombin; lane 2, wt-rPCI; lane 3, wt-rPCI and thrombin; lane 4, P1-rPCI (R354A); lane 5, P1-rPCI and thrombin, lane 6, P14-rPCI (T341A); lane 7, P14-rPCI and thrombin.

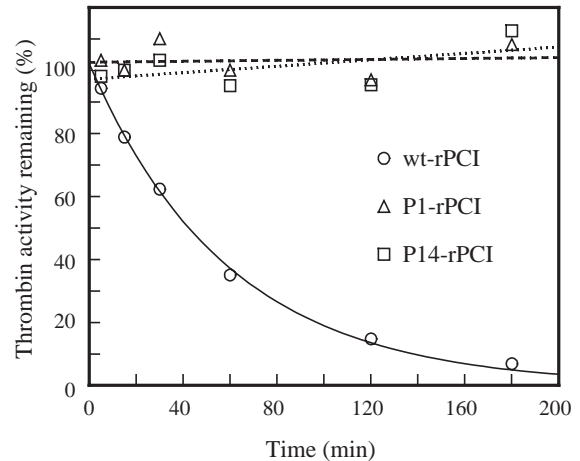


Fig. 4. Thrombin inhibition by rPCI, P1-rPCI and P14-rPCI in the absence of heparin. Thrombin and rPCI molecules were incubated as described under Materials and methods and the remaining thrombin activity was assessed. Results are expressed as the average of at least three independent assays done in triplicates.

proteins appeared as single bands (>90% pure) (Fig. 3A, lanes 2, 4 and 6). Thrombin formed a SDS-stable bimolecular complex with wt-rPCI (Fig. 3A, lane 3) but not with either P1-rPCI or P14-rPCI (Fig. 3A, lanes 5 and 7). Using the anti-His-tag antibody, we verified that all three rPCI proteins had the His-tag and that wt-rPCI formed a stable bimolecular complex with thrombin (Fig. 3B, lane 3) whereas P1-rPCI and P14-rPCI do not interact with thrombin to form a stable complex (Fig. 3B, lanes 5 and 7). These results (stable complex formation between wt-rPCI and thrombin) were further confirmed by immunoblot using the PCI monoclonal antibody (Fig. 3C, lane 3). The appearance of a lower M_r species of rPCI revealed by the PCI monoclonal antibody but not by the anti-His-tag antibody suggests that thrombin cleaves off some of the His-tag at the thrombin site engineered in the pET-15b vector immediately upstream of the amino-terminus of PCI (compare Fig. 3B to C, lanes 3, 5, and 7). As expected since it is a substrate serpin, P14-rPCI is more susceptible to proteolysis by thrombin (Fig. 3C, lane 7) than either wt-rPCI or P1-rPCI (Fig. 3C, lanes 3 and 5).

Table 1

Second-order rate constants (k_2) for the inhibition of thrombin, activated protein C and urokinase by wild-type rPCI in the absence and presence of heparin

Protease	Inhibition rate constant, k_2 ($M^{-1} \text{min}^{-1}$) ^a		
	–Heparin	+Heparin (10 $\mu\text{g}/\text{mL}$)	–Fold increase
Thrombin	$5.67 \pm 0.22 \times 10^5$	$14 \pm 3.0 \times 10^6$	25
Activated protein C	$3.4 \pm 0.7 \times 10^4$	$2.4 \pm 0.7 \times 10^6$	71
Urokinase	$2.3 \pm 0.3 \times 10^4$	$6.1 \pm 0.3 \times 10^6$	265

^a The results are expressed as the average of at least three independent experiments done in triplicates.

3.4. PCI inhibition of proteases in the absence and presence of heparin

To assess the functional integrity of rPCI and its inactive mutants, protease inhibition assays were initially performed using thrombin (Fig. 4). With wt-rPCI there is a progressive loss of thrombin activity whereas there was no thrombin inhibition either by P1-rPCI or by P14-rPCI, even after an 8-h incubation (Fig. 4). These results give further evidence

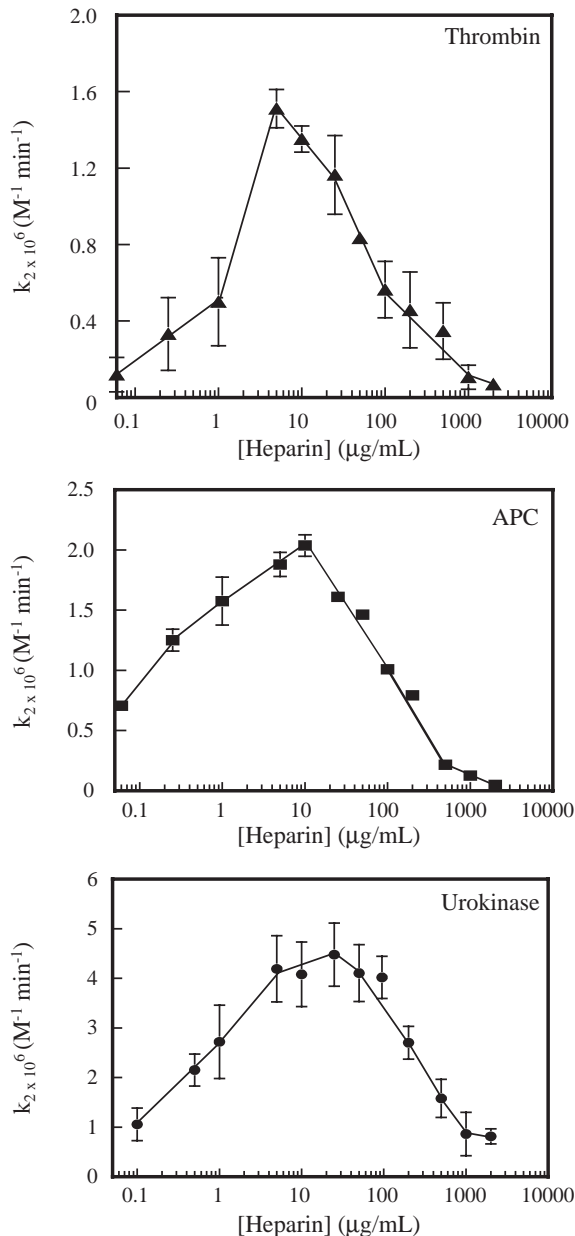


Fig. 5. Inhibition of thrombin, APC and urokinase by wt-rPCI in the presence of heparin. Reactions were performed by incubating 1 nM thrombin (upper panel), 1 nM APC (middle panel) or 2 nM urokinase (bottom panel) with wt-rPCI (10-fold excess to protease) and heparin in the activity buffer of each protease as detailed in Materials and methods. Results express the average of at least three independent experiments done in duplicates.

that both the P1 and P14 rPCI mutants have lost their inhibitory activity. With a k_2 value of $5.67 \pm 0.22 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ (Table 1), thrombin inhibition by wt-rPCI compares favorably with the k_2 for human plasma purified PCI (k_2 , $4.8 \pm 0.12 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$, data not included). Likewise, wt-rPCI inhibition of both urokinase and APC gave inhibition rate constants consistent with that of plasma-derived PCI (Table 1).

The number of serpin molecules consumed before an inactivated complex is formed is shown in its stoichiometry of inhibition (SI). The SI value for wt-rPCI with thrombin was 2.0 (data not included), comparable to what has been previously found for human plasma PCI [32].

Heparin (10 $\mu\text{g/mL}$) increases the rate of thrombin inhibition wt-rPCI by 25-fold ($1.4 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$), which compares favorably to the 34-fold increase in k_2 ($1.6 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$) with plasma-derived PCI. As expected, heparin did not have any effect on the inability of P1-rPCI and P14-rPCI to inhibit thrombin (data not shown). Likewise, there was a 71- and 265-fold enhancement of the rate with heparin (10 $\mu\text{g/mL}$) for APC and urokinase by wt-rPCI, respectively (Table 1). Heparin concentration inhibition curves for rPCI showed that at optimal concentrations of heparin, the k_2 values for the inhibition of thrombin, APC and urokinase were $1.51 \pm 0.1 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ at 5 $\mu\text{g/mL}$ heparin, $2.03 \pm 0.09 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ at 10 $\mu\text{g/mL}$ of heparin, and $4.48 \pm 0.64 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ at 25 $\mu\text{g/mL}$ of heparin, respectively (Fig. 5).

4. Discussion

This study describes for the first time a protocol for the expression and purification of human rPCI, and two inactive rPCI mutants, produced in *E. coli*. The expression and purification of rPCI from the BL21(DE3)pLysS *E. coli* strain was achieved in about 4 days, starting with an overnight mini-culture of bacteria to removing rPCI from dialysis for activity measurements. Recombinant wild-type PCI has similar inhibitory activity to plasma-purified PCI with thrombin whereas the mutant P1-rPCI and P14-rPCI were not inhibitory. The formation of a covalent complex between PCI and thrombin was confirmed by SDS-PAGE analysis. Similar wt-rPCI inhibition results were obtained with both urokinase and APC. In a different expression system, *N*-linked glycosylation rPCI mutants had either enhanced or reduced protease inhibitory activity [39]; however, this is apparently not an issue as described here for bacterially-expressed rPCI. The absence of PCI glycosylation does not interfere either with heparin binding or with inhibitory activity when compared to plasma-purified PCI using APC, urokinase and thrombin as the target proteases; thus, we cannot confirm the interesting results of Fujita et al. [39]. The recent PCI-APC-heparin studies reporting inhibition rate increases approaching 1000-fold most likely represent different inhibition assay conditions

including the source of heparin [36,40], since the heparin-catalyzed APC inhibition rates for bacterially-expressed rPCI were virtually identical to our past studies with plasma-derived PCI. SI values for wt-rPCI with thrombin were essentially the same in wt-rPCI from *E. coli* compared to human plasma-derived PCI. Protein C inhibitor likely has a higher SI value than the prototypic serpin, α_1 -antitrypsin, because it can be often cleaved without forming a stable protease-PCI complex. The close k_2 and SI values and formation of bimolecular complexes suggest that rPCI from *E. coli* is a good model for human plasma-derived PCI for further in vitro and in vivo experiments.

One issue we encountered using *E. coli* to express rPCI was the co-purification of GroEL proteins tightly associated to rPCI molecules, when co-eluted from the nickel column. First, we found that performing protein expression at 30 °C instead of 37 °C reduced the amount of GroEL protein associated to rPCI molecules, either by decreasing GroEL expression or by reducing the amount of denatured rPCI molecules recruiting GroEL proteins for correct folding. Next, we modified our purification protocol by using either heparin-Sepharose chromatography or by using a more precise imidazole elution profile, which allowed us to recover rPCI to a higher degree of purity. These findings suggest that chaperone proteins are necessary for rPCI molecules to correctly fold in this *E. coli* expression system [41,42].

Soluble PCI can be obtained by performing the purification of the serpin from human blood or urine and purified PCI has been used for in vitro experiments to identify its target proteases [1,3,4,25,43]. However, the introduction of mutations into rPCI should allow for the identification of residues important for the biochemical properties of PCI. Several rPCI mutants within the reactive site loop and in heparin binding site have previously been generated using the baculovirus/insect cell system [10,19,31–35,44]. Although this system is advantageous because of its ability to perform most of the post-translational protein modifications and that it allows for proper folding of recombinant proteins, insect cell expression systems are less easily manipulated than bacterial systems are, they are more difficult to culture and utilize on a routine basis, and the protein yields are typically less than the usual bacterial expression systems. Thus, an ideal serpin expression system would require only a few days to produce protein, provide milligram quantities of proteins, employ a simple purification scheme, and allow other serpin scientists a useful procedure to study rPCI. Some serpins including α_1 -antitrypsin (*SERPINA1*), plasminogen activator inhibitor-1 (*SERPINE1*), plasminogen activator inhibitor-2 (*SERPINE2*), α_1 -antichymotrypsin (*SERPINA3*), kallistatin (*SERPINA4*), C1 inhibitor (*SERPIND1*), MENT (myeloid and erythroid nuclear termination stage-specific protein), and heparin cofactor II (*SERPIND1*) have been expressed using a bacterial system and displayed essentially normal biochemical activities [45–52].

Serpins are of major interest to many different scientists and clinicians since they locally regulate the proteolytic activities of serine (and some cysteine) proteases that are involved in a broad range of physiological processes. Loss of this regulation of serine proteases activity by serpins is associated with pathological situations such as thrombosis, emphysema, sterility, and cancer [16,20,53,54]. Several other studies have found an additional role for serpins in cell adhesion, proliferation, invasion, apoptosis, and angiogenesis [19,55–58].

Protein C inhibitor was first purified from human plasma in 1983 and was identified as an inhibitor of both APC and thrombin [1]. The role of PCI in coagulation is confusing since the inhibition of APC by PCI supports further thrombin formation [59–61], and since PCI is a potent inhibitor of thrombin when bound to thrombomodulin [11,12]. These opposing actions suggest that PCI behaves both as a pro-coagulant and as an anti-coagulant serpin, although its role in regulating coagulation and fibrinolytic proteases is still not fully understood. Protein C inhibitor has also recently been shown to regulate male spermatogenesis but the mechanism by which PCI participates in this process is still under active investigation [16,62–65]. It is important to have murine models to better define the in vivo role of human PCI [14–16]. However, a difficulty in studying the in vivo role of human PCI in a vascular biology setting is the absence of liver-produced and circulating murine PCI [14–16]. Thus, having a complementary in vitro approach to assess the biochemical/physiological roles of human PCI with this recombinant *E. coli*-based expression system might help in this context.

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