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Renaturation, Purification, and Characterization of Human Plasminogen Activator Inhibitor Type 2 (PAI-2) Accumulated at High Level in *Escherichia coli*

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Plasminogen activator inhibitor 2 (PAI-2) is an important regulator of plasminogen activation, which inhibits both tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). In this study we have developed a high-level expression system by inserting a modified PAI-2 gene downstream of the T7 promoter. The expression level of recombinant PAI-2 amounted to 55-60% of total microbial protein. By efficient renaturation and one-step purification, the recombinant protein was purified to homogeneity. The specific activity and yield of recombinant PAI-2 reached 33,000 IU/mg and 10 mg per gram wet weight of *Escherichia coli* cells, respectively. The second-order rate constant for uPA was $2.6\text{-}2.8\times10^6$ $M^{-1}\cdot s^{-1}$.

Key words: gene expression, inclusion body, plasminogen activator inhibitor 2 (PAI-2), renaturation, serine protease inhibitor (SERPIN).

Plasminogen activators (PAs) play a central role in the regulation of the fibrinolytic system in blood. Their activities are controlled by specific inhibitors, PAIs. Four different PAIs have been identified: the endothelial cell type PAI (PAI-1), the placenta type (PAI-2), the urinary type (PAI-3), and protease nexin 1 (*1-4*). PAs and PAIs participate in many important physiological reactions, such as fibrinolysis, inflammation, and tissue remodeling, and may play roles in tumor growth and metastasis (*5, 6*).

As a member of the superfamily of SERPINS (serine protease inhibitors), PAI-2 is an efficient inhibitor of uPA and two-chain form tPA (*7*). It can be synthesized in macrophages and epithelial cells and it is abundant in human plasma late in pregnancy (*8, 9*). The monocytoid cell line U937 also can produce PAI-2 (*10*). The PAI-2 protein exists in two forms: an intracellular, non-glycosylated form of 47 kDa and an extracellular, glycosylated form of about 58-60 kDa (*3, 7*). The amino acid sequence of PAI-2 reveals that it has significant homology to chicken ovalbumin (*11*). It has an internal signal which allows the bitopological distribution of the protein (*12*). The relative distribution of intracellular and secreted forms of PAI-2 also seems to depend on the cell type, culture conditions, and differentiation state of the cell (*11-13*). Recently Mikus and Ny have reported that PAI-2 readily polymerizes intracellularly and the polymerization may lead to reduced secretion efficiency (*14*).

Natural sources of PAI-2 yield extremely small quantities of purified material (*3, 7, 15*). Many attempts have been made to express the protein in *Escherichia coli* and mammalian cells (*10, 16-18*), but most of them have been disappointing. Here we describe the high-level expression of recombinant unglycosylated PAI-2 in *E. coli* and the procedures for its purification.

MATERIALS AND METHODS

Materials—Restriction endonuclease, T4 DNA ligase, and calf intestinal alkaline phosphatase were purchased from Promega and New England Biolabs. These enzymes were used in accordance with the instructions of the suppliers. The PCR kit and T7 DNA sequencing kit were purchased from Promega. The chromogenic plasmin substrate S-2444 (Pyr-Glu-Gly-Arg-pNA) was purchased from Sigma. Q-Sepharose was a product of Pharmacia. Rabbit antiserum to human PAI-2 was kindly provided by Doctor Cao Xiangrong. The high-molecular-weight (HM_r) uPA (100,000 IU/mg) was a generous gift from Professor Dexu Zhu.

Bacteria and Plasmids—*E. coli* BL21 (DE3), which contains an integrated copy of the T7 RNA polymerase gene, was used as the host for expression plasmids. *E. coli* DH5α was used for cloning plasmids. Plasmid pET11d was used as the expression vector and the PAI-2 cDNA was obtained from the plasmid pEM-PAI-2. The expression vector pET11d was first digested with *Bam*HI and blunted with Klenow fragment, then the linear plasmid was digested with *Nco*I. Because there are two *Xmn*I endonuclease sites in the encoding sequence of PAI-2, it is difficult to cut off most of the 3'-untranslated sequence from PAI-2 cDNA directly, so we adopted the method of Zeng *et al.* (*19*) to obtain the entire encoding sequence from plasmid pEM-PAI-2, except for the first two amino acid residues. First the plasmid pEM-PAI-2 was digested with *Bgl*II, *Xba*I, and

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Abbreviations: PA(s), plasminogen activator(s); PAI(s), plasminogen activator inhibitor(s); uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; TNF-b, a mutant of tumor necrosis factor α; BSA, bovine serum albumin; DTT, dithiothreitol.

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*Eco*RI, and both fragments of PAI-2 cDNA were recovered separately. Then the large fragment was digested with *Xmn*I to cut off the 3′-untranslated sequence. To clone PAI-2 gene into the expression vector, two oligonucleotides, 5′-CATGGAG-3′ and 5′-GATCCTC-3′, encoding the two N-terminal amino acids of PAI-2, were synthesized. Finally, the two encoding sequences of PAI-2 gene and the adapter, about 1.3 kb in total length, were ligated into the expression vector pET11d to create the expression plasmid pET-PAI-2. The construction was produced by standard techniques, and the procedure is illustrated in Fig. 1.

Production of PAI-2—Transformants harboring the expression plasmid pET-PAI-2 were incubated in LB medium supplemented with ampicillin (50 μg/ml) at 37°C overnight and then the cultures were inoculated in fresh LB medium with ampicillin (25 μg/ml) at a ratio of 1:50. Two hours later, a part of the culture was taken for SDS-PAGE and expression of recombinant PAI-2 was induced with IPTG (0.01 mM). After 10 h of incubation the cells were harvested, sonicated, and centrifuged to obtain the supernatant and the pellet. The activity in the supernatant was measured.

Renaturation and Purification of Recombinant PAI-2 from E. coli—The sonicated pellets were washed twice with 50 ml of washing buffer (50 mM Tris-HCl, pH 8.0, containing 2.0% Triton X-100, and 5 mM EDTA). The final pellet gave inclusion bodies containing the recombinant PAI-2 (rPAI-2). The inclusion bodies (about 100 mg) were solubilized in 10 ml of denaturation buffer (50 mM Tris-HCl, pH 8.0, containing 8 M urea, and 20 mM 2-mercaptoethanol) with stirring for 2 h at room temperature. After centrifugation, the supernatant was slowly dropped into 1 liter of renaturation buffer (2 M urea, 50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM reduced glutathione, and 0.2 mM oxidized glutathione), to a final protein concentration of less than 0.1 mg/ml, and vigorously stirred. The solution was kept at 4°C for 24 h and PAI-2 activity was measured. The solution was centrifuged at 10,000 × *g* for 20 min, then the supernatant was diluted 5 times with buffer A (20 mM Tris-HCl, pH 8.0, 1 mM DTT). The sample solution was applied to a Q-Sepharose column (1.5 cm × 25 cm) previously equilibrated with buffer A. The bound protein was eluted with an increasing concentration gradient of NaCl from 0-0.5 M. The active fractions were pooled, dialyzed against 5 mM Tris-HCl (pH 8.0), and lyophilized.

SDS-PAGE and Western Blotting—Electrophoresis was performed under reducing conditions (*20*). Western blotting was performed as described (*16*).

PAI-2 Activity Assay—The inhibitory activity of PAI-2 was measured by determining the inhibition of uPA activity using a synthetic substrate, S-2444. A 50 μl sample and 2 U of uPA in 50 μl of activity assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, containing 0.01% Tween 80, and 100 μg/mg BSA) were incubated in microtiter plates. After 30 min incubation at room temperature, 100 μl of 0.5 mM S-2444 was added and residual uPA activity was quantitated by measuring the change in absorbance at 405 nm at 15 min intervals in a Titertek multiscan spectrophotometer. One inhibitory unit was defined as the amount of PAI-2 required to inhibit one Ploug unit of urokinase.

Kinetic Analysis of the Interaction of uPA with Recombinant PAI-2—The second-order rate constant for the interaction between rPAI-2 and uPA was determined in a single-step assay as described (*21*). The rate constant was calculated using the following form of the standard second-order rate equation:

$$k_i t = (b/a)\ln\{(a[E]/b + [E]_0[E])/(a[E] + [E]_0[E])\} \quad (1)$$

where a is $([I]_0 - [E]_0)$, b is $(1 + [S]/K_m)$, $[E]_0$ is the initial concentration of enzyme, [E] is the concentration of free enzyme at time t, $[I]_0$ is the initial concentration of PAI-2, [S] is the substrate concentration and the K_m is the appropriate Michaelis-Menten constant for the substrate and enzyme. The concentration of free enzyme was calculated by using equation:

$$[E] = T[E]_0/bT_0 \quad (2)$$

where T_0 is the slope of the plot (the change in absorbance *versus* the change in time in the absence of PAI-2) and T is the slope at time t in the presence of PAI-2. Reactions were carried out at 25°C in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, and 0.01% Tween 80. PAI-2 and substrate were mixed (0.1 ml) and placed in a spectrophotometer. At t_0, 0.1 ml of uPA solution was added and the change in absorbance at 405 nm was recorded with time. The K_m of uPA for the substrate S-2444 is 0.059 mM.

Assay of Complex Formation between PAI-2 and uPA—A mixture of 5 μg purified rPAI-2 and 10 μg HM_r uPA was incubated at room temperature for 10 min. Then the sample was subjected to SDS-PAGE under reducing conditions, followed by silver staining analysis.

Protein Determination—Protein concentrations were measured by the method of Bradfold (*22*) using BSA as a standard.

RESULTS

Construction and Characterization of PAI-2 Expression Plasmid—By using the *Xba*I site in the encoding sequence of PAI-2 cDNA, most of the 3′-untranslated sequence was removed and the resultant PAI-2 gene was cloned into expression plasmid pET11d. The construction procedure is illustrated in Fig. 1. The recombinant expression plasmid

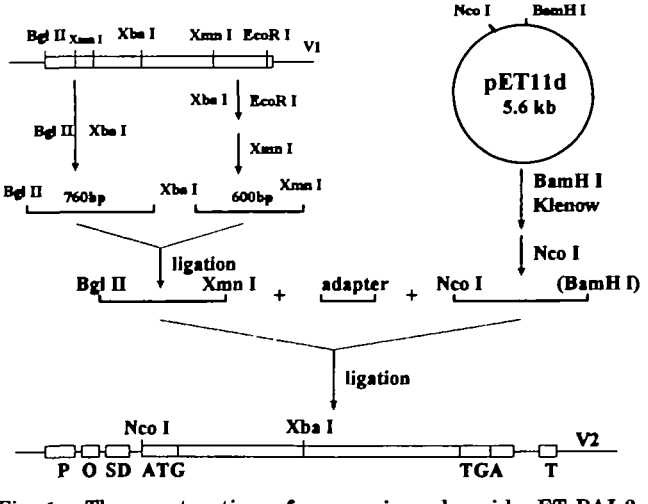

Fig. 1. **The construction of expression plasmid pET-PAI-2.** Abbreviations: P, T7 promoter; O, lac operator; T, T7 terminator; SD, Shine-Dalgarno sequence; ATG, translational start codon; TGA, translational termination codon. V1, pEM-PAI-2; V2, pET-PAI-2.

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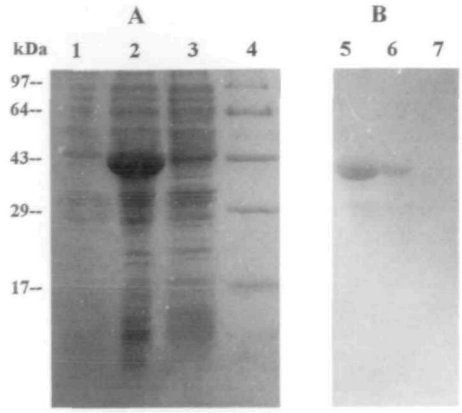

Fig. 2. **SDS-PAGE.** (A) Coomassie Brilliant Blue R-250 staining gel. (B) Western blotting. Lanes 1 and 7, pET11d in *E. coli* BL21 (DE3); lanes 2 and 5, pET-PAI-2 in BL21 (DE3) induced; lanes 3 and 6, pET-PAI-2 in BL21 (DE3) uninduced; lane 4, molecular mass standards, with masses indicated in kDa.

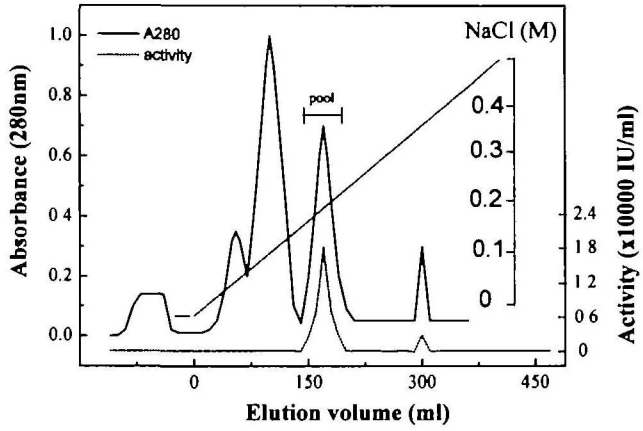

Fig. 3. **Purification of active recombinant PAI-2 by Q-Sepharose column chromatography.** The renaturation solution was applied to a Q-Sepharose column (1.5 cm × 25 cm). The bound protein was washed with a 400 ml linear gradient in the range of 0-0.5 M NaCl in 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM DTT (2.0 ml/min).

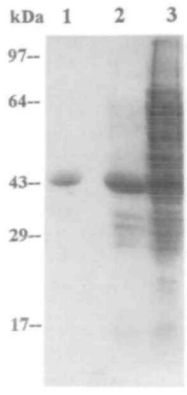

Fig. 4. ***Characterization of recombinant PAI-2 by Coomassie* Blue-staining SDS-PAGE during purification.** Lane 1, purified rPAI-2; lane 2: the inclusion bodies; lane 3, the supernatant of the expression cell lysate.

ABLE I. **Purification of recombinant PAI-2 from 2 g wet eight of *E. coli* cells.** The data shown are from a single purification ocedure, typical of five replicates.

Step	Volume (ml)	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Yield (%)
lenaturation solution	1,000	87.0	750,000	8,621	100
-Sepharose eluate	55	19.8	660,000	33,333	88.0

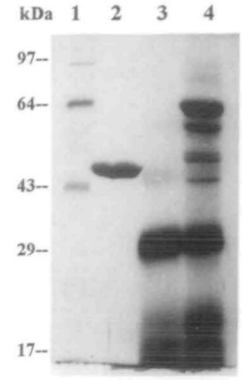

Fig. 5. **SDS-PAGE analysis of complex formation between recombinant PAI-2 and HM_r u-PA.** The procedure was as described in "MATERIALS AND METHODS." After electrophoresis, the gel was silver stained. Lane 1, molecular weight marker; lane 2, purified recombinant PAI-2; lane 3, HM_r uPA; lane 4, HM_r uPA and rPAI-2. All samples were reduced in sample buffer containing 20 mM DTT and analyzed by SDS-PAGE in 10% polyacrylamide gel.

(pET-PAI-2) was confirmed to be as expected by enzyme digestion and partial DNA sequencing (date not shown).

Productivity of Recombinant PAI-2 in E. coli—The transformants containing the plasmid pET-PAI-2 were cultivated and assayed by SDS-PAGE to estimate the amount and size of the recombinant PAI-2 produced in the total cell lysates. rPAI-2 was detected on Coomassie Brilliant Blue R-250-stained gel as a distinct band with the mobility of about 47 kDa after induction by IPTG (Fig. 2A). rPAI-2 can also be expressed without induction. Immunoblotting with the polyclonal anti-human PAI-2 antibody confirmed the identity of these peptide bands (Fig. 2B, lanes 5 and 6). The expression level of rPAI-2 was measured by densitometric scanning of Coomassie Brilliant Blue R-250-stained gel and the amount of expressed PAI-2 was about 60% of the total cellular proteins. The PAI-2 activity was detected in the cell lysate from expression cultures, but most of the rPAI-2 was in the form of inclusion body (Fig. 4, lane 2).

Renaturation and Purification of Recombinant PAI-2—Since rPAI-2 is expressed in *E. coli* mainly in an insoluble, denatured form, we developed a protocol that allowed the recovery of significant amounts of active rPAI-2. The inclusion bodies were washed with the washing buffer and analyzed by SDS-PAGE, which indicated that about 85% of the protein was rPAI-2 (Fig. 4, lane 2). After renaturation, the supernatant of the renaturation solution was diluted and loaded onto a Q-Sepharose column (Fig. 3). Most of the soluble rPAI-2 without activity passed through the column and the fractions of the large peak before the active

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fractions contained little protein but mostly nucleic acid contaminants (data not shown). The fractions eluted near 0.20 M NaCl with PAI-2 activity were pooled, dialyzed and lyophilized. In this way the rPAI-2 was purified to homogeneity (Fig. 4, lane 1) with a specific activity of up to 33,000 IU/mg. The one-step purification procedure for rPAI-2 is summarized in Table I.

Inhibition of uPA with Recombinant PAI-2—The purified rPAI-2 was subjected to SDS-PAGE. There was only one band in the gel stained by Coomassie Brilliant Blue R-250 (Fig. 4, lane 1) as well as by silver (Fig. 5, lane 2). The specific activity of rPAI-2 and the second-order rate constant for uPA were about 3.3×10^4 IU/mg and $2.6\text{-}2.8 \times 10^6$ $M^{-1} \cdot s^{-1}$, respectively.

To study complex formation, the recombinant PAI-2 was mixed with excess HM_r uPA and analyzed with SDS-PAGE, followed by silver staining. As shown in Fig. 5 (lane 3), under reducing conditions the HM_r uPA shows a well-defined band that migrated with an apparent $M_r = 33{,}000$ and a fuzzy zone just below $M_r = 22{,}000$. Theoretically, as the active site of uPA is located in the heavy chain (33 kDa) and after complex formation, the C-terminal of PAI-2 was removed, the molecular weight of the SDS-stable complex should be about 75 kDa. Our result shown in Fig. 5 (lane 4) is consistent with this. Also it can be seen from the figure that almost all purified PAI-2 was active.

DISCUSSION

The method presented above for high-level expression and purification of recombinant PAI-2 from prokaryotic *E. coli* cells is superior to those reported previously (*11, 16-18*) in several respects: firstly, the expression level of rPAI-2 reached 55-60% of the total cell protein, which is much higher than those of the previous reports. The high-level expression of PAI-2 may be due to the deletion of 3′-untranslated region of the gene and the fact that pET serial expression vectors are suitable for expression of some eukaryotic genes. In our laboratory, several genes such as TNF-b (a mutant of tumor necrosis factor α, with more basic amino acids) (*23*), nm-23 (*24*) and PAI-1 (*25*) have been successfully expressed in these vectors at levels between 20-80% of total cellular protein. Secondly, only one anion-exchange chromatography step is needed for purifying rPAI-2 to homogeneity, with the specific activity of purified rPAI-2 reaching 33,000 IU/mg, almost 3 times that of Mikus *et al.* (*18*), and the yield reaching 10 mg of rPAI-2 per gram of wet cells. This true bench-top procedure does not depend on prepackaged columns or high-pressure chromatography and can be easily scaled up or down. Thirdly, in some previous reports (*16-18*), the purified rPAI-2 existed in two forms: 47 kDa and a protease-digested form of 42 kDa. But in our case, no protease digestion was detected and almost all the purified rPAI-2 is about 47 kDa in molecular weight. The reason, we think, may be that in our procedure, the recombinant protein is produced mostly in the form of inclusion bodies and after washing with the washing buffer, little protease contamination from *E. coli* remained. Thus, we conclude that our method for preparing large quantities of active rPAI-2 is efficient and convenient.

Interestingly, we have found that the rPAI-2 can be expressed without induction by IPTG and the expression level of an overnight culture (without induction) reached 50% of total cellular protein, which is a little lower than that induced by IPTG. This phenomenon has also been observed in the expression of several other genes in our laboratory. When cloned into the same expression vector, some genes such as thromobopoietin (*26*), PAI-1 (*25*), and M-CSF (unpublished data) are expressed in *E. coli* cells only after induction with IPTG, but induction is unnecessary for several other genes such as TNF-b (*23*) and nm23 (*24*) with rather high expression levels (more than 40% of total cellular protein). The reason for this is unclear. Since the three different genes (PAI-2, TNF-b, and nm23) can all be expressed by *E. coli* cells without induction, we think that they may have similar regulation mechanisms. Further investigation is in progress.

The availability of active PAI-2 in large quantity will facilitate further investigation of PAI-2's role in both physiological and pathophysiological processes. Also PAI-2 can be used to study the relationship between the inhibitor's function and structure. Further study is in progress to investigate PAI-2 inhibition of the invasion and metastasis of tumor cells in mice.

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