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Cloning, Expression, and Renaturation Studies of Reteplase

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Abstract Recombinant human tissue plasminogen activator deletion mutein (Reteplase) is a clinically promising thrombolytic drug. Reteplase cDNA was subcloned into a bacteria expression system, and the resultant recombinant was biologically characterized. The Reteplase was expressed in *Escherichia coli* as an inclusion body, and the downstream processes of the Reteplase inclusion body included denaturation, renaturation, and purification. A protein disulfide isomerase (PDI) was used to assist the refolding of Reteplase, and it was found to increase the refolding rate from less than 2% to more than 20%. The refolded Reteplase was purified through two chromatography steps, including lysine-coupled agarose affinity chromatography and then CM-sepharose cation-exchange chomatography. The purity of r-PA was analyzed by Western bolt analysis, and N-terminal amino acid and amino acid composition analyses confirmed the end-product. Reteplase showed higher thrombolytic potency in an animal thrombus model.

Key words: Reteplase, cloning and expression, renaturation, protein disulfide isomerasse

Cardiovascular and cerebrovascular diseases are the leading life-threatening diseases in the world. Over twelve million patients worldwide die of the disease each year as estimated by WHO. The mortality rate exceeds two million people each year in China, while at least three million patients need treatment with thrombolytic drugs. It is worth to note that coagulation distinction or thrombus formation is widely involved in many disorders of the liver, kidney, and endocrinologic metabolism as well as common diseases in surgery, gynecology, and gerontology, not to mention stroke, myocardial infarction, and disseminated intravascular coagulation. It is therefore in urgent demand to explore thrombolytic mechanisms and manufacture effective thrombolytic drugs. Recombinant tissue plasminogen activator deletion mutein (Reteplase, abbreviated as r-PA) represent one of the third generation thrombolytic drugs. Reteplase manifests advantages in large-scale production, prolonged half-time, thromobolytic potency, and fewer side effects, thus it attracts great attention from cardiovascular experts [6, 16].

Reteplase has been obtained by deleting the three structural domains of Kringle I, Finger, and EGF, while retaining the thrombolytic Kringle II, protease domain, two functional regions of protease (amino acid residues 176-527), as well as the N-terminal 1 to 3 amino acid residues [8, 10, 12, 17]. Reteplase expressed in *Escherichia coli* is in the form of an inclusion body and needs to be renatured to gain biological activity. Since there are 9 disulfide bonds in the r-PA molecule, renaturation is very difficult. Therefore, in this study, the cDNA of r-PA was cloned, and r-PA was expressed in *Escherichia coli* and renatured. The effect of PDI on r-PA renaturation was investigated, which had never before been reported by other scientists.

Plasmid pET22b was used as the vector, which has the T7 promoter and terminator and is ampicilin resistant (Novagen Co., Santee, CA, U.S.A.). *E. coli* BL21(ED3) was the host. The human brain cDNA (Clonetech Co., Palo Alto, CA, U.S.A.) was the template for the PCR. The following two PCR primers were designed for the cloning of r-PA cDNA: primer 1: 5'-GGGGGCATATGTCTTA-TCAGGGAAACAGTGACTGCTAC-3', and primer 2: 5'-GGGGCTCGAGTCACGGTCGCATGTTGTC-3'. The protein disulfide isomerase (PDI) with a purity more than 95% was used in the experiment.

Expression of r-PA was performed using a 300-ml flask, containing 100 ml of LB medium. The inoculation volume of 1% was cultivated at 37°C, and shaken for 14 h at 150 rpm. Isopropyl-1-thio-β-galactopyranoside was added to the final concentration of 1 mM in the middle of the exponential growth phase. During recovery of the inclusion body, the cells were suspended using Tris-EDNA buffer, disrupted using sonication (Cole-Parmer, Vernon Hills,

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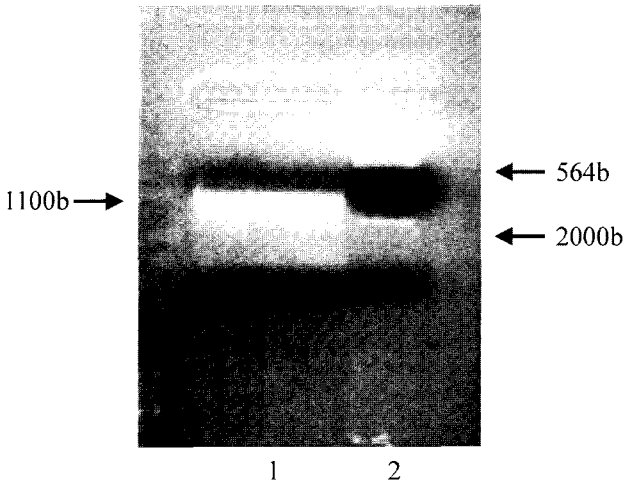

Fig. 1. Agarose gel electrophoresis of the PCR amplified fragment. Lane 1, fragment (1,100 bp); lane 2, DNA molecular weight markers.

Illinois; treated with 100 ml for 3 sec per treating, a total of 150 times), and centrifuged at 15,000 ×g for 20 min. The sediment was washed and recentrifuged. Reteplase was purified using a lysine-coupled agarose affinity chromatography and a CM-sepharose cation-exchange chromatography (Pharmacia Co., Sweden). The amino acid sequence was analyzed by the amino acid sequence analyzer of an ABI-Precise-491 (Applied Biosystems Co., Foster, CA, U.S.A.). Molecular weight was analyzed by MALDI-MS (Bruker Co., Germany). The r-PA activity was measured according to the method of Verheijen *et al.* [19]. The purity of r-PA was measured by protein C4 RP-HPLC and silver staining SDS-PAGE.

The r-PA cDNA was cloned using PCR with primers and the template, as described above. An amplified fragment of 1.1 kb in size was obtained (Fig. 1), cloned into the vector of pUC19, sequenced, and confirmed to contain the r-PA cDNA sequence of 1,065 bp coding for 355 amino acids which has been reported previously [8] (Fig. 2). After the plasmid of pET22b was digested using restriction enzymes *Nde*I and *Xho*I, the amplified fragment was inserted into the plasmid pET22b, yielding the plasmid pTPA3. This plasmid was confirmed to have the anticipated strucure by enzymatic restriction analysis (data not shown). Transformation of *E. coli* BL21 (DE3) using pTPA3 yielded the recombinant r-PA producer pTPA/BL21. SDS-PAGE analysis showed that the expressed product of pTPA3/BL21 had a molecular weight of approximately 39 kD (Fig. 3). Thin-layer chromatography scanning analysis showed that the product was about 40% of the total soluble proteins producted (data not shown).

Renaturation was carried out in order to obtain the bioactive product. This process included (1) denaturalization and dissolution of r-PA in the inclusion bodies, and (2) renaturation of the dissolved r-PA to obtain the correctly folded product. The generally used 3 M guanidine hydrochloride concentration in denaturalization and dissolution can dissolve very little r-PA, because the 18 hydrosulfide groups in the r-PA molecule form numerous mismatched disulfide bonds and make the r-PA inclusion body very tight. In this experiment, 6 M guanidine hydrochloride and

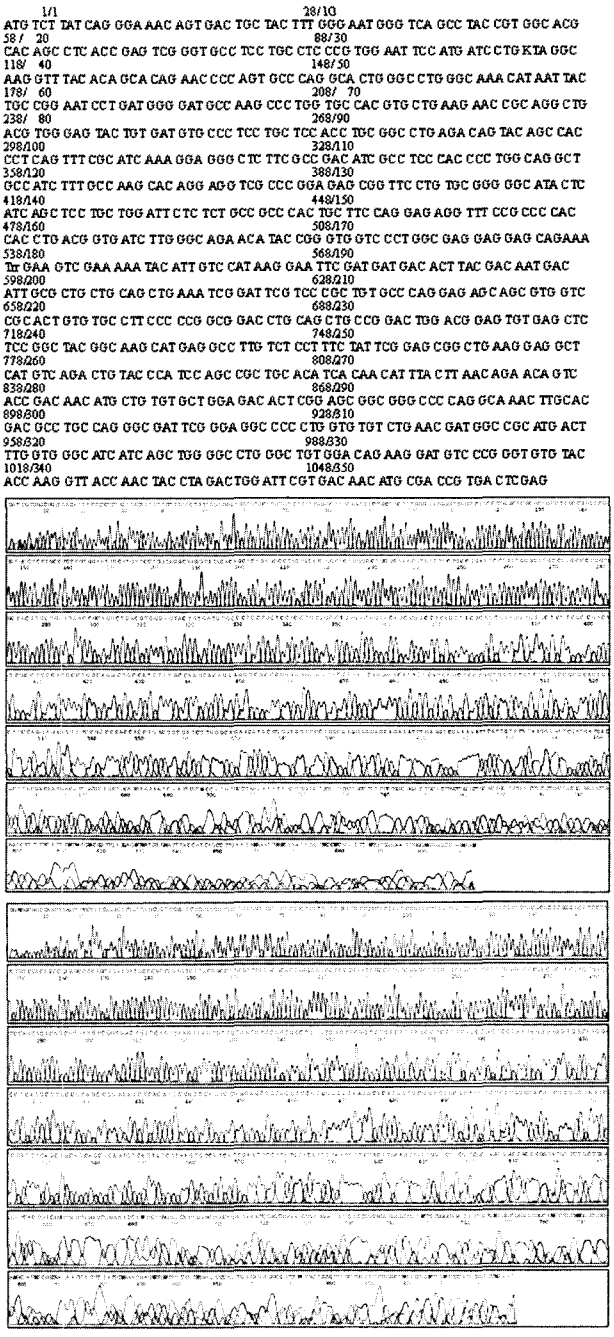

Fig. 2. The cDNA sequences analysis of Reteplase.
Computer read sequence, both from 5'→3' (middle) and 3'→5' (bottom). This result, which was confirmed to contain the r-PA cDNA sequence of 1,065 bp coding for 355 amino acids, was the same as that reported previously by Kohnert *et al.* [8] (top).

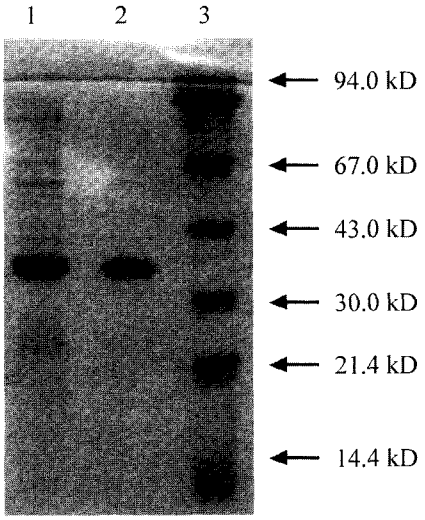

Fig. 3. SDS-Page analysis of the expressed r-PA.
Lane 1, post-induction; lane 2, purified inclusion (39 kD); lane 3, molecular weight markers.

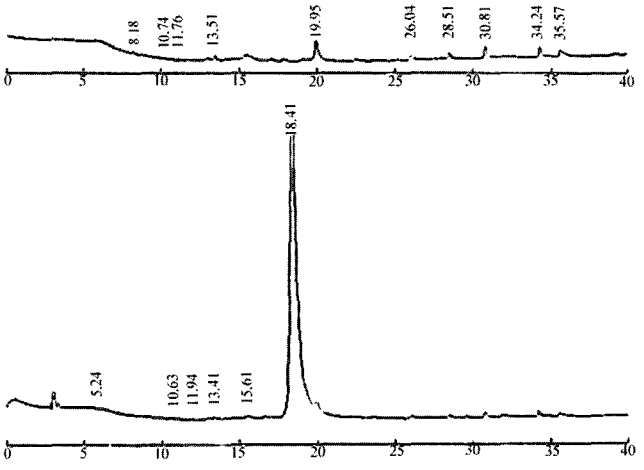

Fig. 4. Purity analysis of the purified r-PA using HPLC.
Top, blank control; bottom, the Reteplase sample was measured three times by C_4 HPLC, and the purity reached 99.41% or more.

100 mM dithiothreitol were used, because through our past experience, they could dissolve and denature more than 80% of the r-PA. In renaturation, the reducing environment was provided by adjusting the ratio of hydrosulfide group/disulfide bond (GSH/GSSG) to 1 mM/0.2 mM in the renaturation buffer. In order to increase the renaturation yield, PDI was added to the renaturation buffer until the mole ratio of PDI/r-PA reached 1/20 and 1/50. The denatured r-PA was slowly added to the renaturation buffer while being stirred. The suspension was stored for 24 h at room temperature (27°C) and filtered to remove the insoluble sediments, thereby yielding the renaturated solution. The r-PA renaturation yield, after PDI was added, reached 25.6% when the molar ratio was 1/20, and 20% when it was 1/50, which was much higher than the yield of 1-2% of the control without PDI addition. During the expression of r-PA in *E. coli*, 18 cysteine residues in r-PA molecule will form 9 disulfide bonds, and chance for the correct matching of the 18 hydrosulfide groups is very low, which results in the low yield of renaturation of the simple oxidative and reductive glutathione system. The addition of PDI, which can specifically catalyze the SH/-SS- exchange reaction [7], opens the mismatched disulfide bonds and facilitates the formation of the correctly matched disulfide bonds, therefore, increasing the refolding rate [13].

Reteplase can be easily separated from PDI, as there are big differences in molecular weight as well as isoelectric points repetition: 57 kD and 4.2 kD, respectively. PDI has been used extensively in improving the folding of a protein with multiple disulfide bonds, for example, RNase with 4 disulfide bonds [4] and prochymosin with 3 disulfide bonds [18]. However, the present study is the first to use PDI to assist protein folding of 9 disulfide bonds [5].

The purification of bioactive r-PA in renaturing solution was carried out by affinity chromatography and ion-exchange chromatography. A lysine-affinity chromatography was employed for separation and purification of r-PA, and it is more stable than protein inhibitor affinity chromatography media [8]. Lysine-affinity media are commercially available and more economical than the protein inhibitor affinity media which are very expensive to prepare. The purity of our product was also higher (99.4% versus 95%), measured by protein C4 RP-HPLC (Fig. 4) and silver staining SDS-PAGE. The specific activity of the separated r-PA was over 5×10^5 IU/mg. N-terminal 15 amino acid sequence, amino acid composition, and molecular weight analyses of the purified product confirmed that the product was r-PA [21].

Experiment involving the intravenous injection of mice with the r-PA showed that thrombus induced by collagen and adrenine could be removed rapidly. The thrombus in the tail induced by carragheenin could be evidently reduced and, although a dosage effect was evident, no hemorrhaging occurred. Finger domain has a high affinity for fibrillarin and results in hemorrhage, thereby it could decrease hemorrhagic side effects when the Finger domain was cut off [20]. The longer half-life in plasma should be one of the reasons for the high thrombolytic ability of r-PA. The Kringle I and EGF domains can combine to liver cell surface receptors and accelerate the clearance of r-PA in plasma [20]. The mannose-rich polysaccharides in the Kringle I domain also cause the clearance of t-PA [14]. Therefore, when the above two domains were cut off, the thrombolytic activity was still maintained [2, 9], the half-life was extended from 5 to 18 min [1, 15], and the dosage per course of treatment in clinics decreased from 100 mg to 17-34 mg [11].

The r-PA without the Kringle I domain has no glycosylation and can be expressed in *E. coli* using high density cultivation,

with high efficiency and low cost, compared with the expression of the glycosylated t-PA using eukaryotes [10].

In conclusion, r-PA is a clinically promising thrombolytic drug with advantages of longer half-life, less hemorrhagic side effects, and especially, a lower production cost. PDI is an effective refolding additive in the folding process of r-PA.

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