

# Construction and expression of anti-CD62P/anti-GPIIb-IIIa diabody

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## ABSTRACT

**الأهداف:** من أجل بناء ظهور ثلاثة من البلازميدات لعلاج تخثر الدم: قطعة متغيرة للسلسلة المفردة (scFv) للجسم المضاد مونوكلونال mAb 7E3 الذي يستطيع تحديد وربط صفائح الجليكوبروتين (GPIIb-IIIa المركب) و (scFv) للجسم المضاد mAb WAPS12.2 الذي يستطيع تحديد وربط الجسم المضاد (CD62P)، وهو الجسم المضاد الذي يمكنه تحديد وربط (GPIIb-IIIa) و (CD62P) بشكل متزامن، ومن أجل فحص ما إذا كانت العوامل يمكنها الظهور بشكل صحيح.

**الطريقة:** أجريت هذه الدراسة في مختبرات شركة هونان يونانتي للتقنية الحيوية المحدودة - الصين، خلال الفترة ما بين سبتمبر 2007 وحتى مايو 2008م. تم الحصول على الحمض النووي من خلايا (RNA) من mAb 7E3 وخلايا WAPS12.2. أجري فحص تفاعل سلسلة الخمائر الناقلة للجلوكون (RT-PCR) من أجل الحصول على الجينات من المستضد المتغير للسلسلة الخفيفة والثقيلة للأجسام المضادة 7E3 و WAPS12.2. تسمى الجينات المستهدفة CD62PVL، 7E3VH، 7E3VL، و CD62PVH. تم الحصول على CD62PVL-CD62PVH و 7E3VL-7E3VH عن طريق تفاعل سلسلة الخمائر الناقلة PCR وربطها مع pET-22b(+). تم تسمية المنتجات pET-scFvCD62P و pET-scFv7E3 بواسطة CD62PVL-7E3VH و 7E3VL-CD62PVH بواسطة تفاعل سلسلة الخمائر الناقلة PCR وتم ربطها مع pET-22b(+). وسميت المنتجات (pET-ED1) و (pET-ED2). أجريت طريقة تفاعل سلسلة الخمائر (PCR) بواسطة أخذ (pET-ED2) كقالب للحصول على الجين المشغل الوراثي بالكامل وتم ربطه مع (pET-ED1). تم تسمية المنتج (pET-7ECD).

**النتائج:** أكد التحديد بواسطة حصر الانشطار داخل النواة وفحص الحمض النووي (DNA) أن حصر ظهور هذه البلازميدات كان ناجحاً. كما أكد الفحص بسولفات دوديسيل الصوديوم (SDS-PAGE) وبقعة وسترن أن هذه البلازميدات تظهر بشكل صحيح.

**خاتمة:** كان ظهور بلازميدات (pET-scFv7E3) و (pET-scFvCD62P) و (pET-7ECD) محصوراً وظاهراً بشكل ناجح، ويؤدي إلى أساس جيد للمزيد من البحث على إدراك الهدف لعوامل التخثر.

**Objectives:** To construct 3 expression plasmids for the targeted therapy of thrombosis: single chain variable

fragment (scFv) of monoclonal antibody (mAb) 7E3 that can identify and bind platelet glycoprotein GPIIb-IIIa complex, scFv of mAb WAPS12.2 that can identify and bind P selectin (CD62P), a diabody that can identify and bind GPIIb-IIIa and CD62P simultaneously, and to investigate whether the vectors can express correctly.

**Methods:** This study was carried out at the Laboratory of Hunan Yuantai Biological Technology Co. Ltd, Hubei, China from September 2007 to May 2008. Total RNA of mAb 7E3 cells and WAPS12.2 cells were obtained. Reverse transcriptase polymerase chain reaction (PCR) was carried out to obtain the genes of variable regions of light and heavy chains of 7E3 and WAPS12.2. Target genes were named 7E3VL, 7E3VH, CD62PVL, and CD62PVH. The 7E3VL-7E3VH and CD62PVL-CD62PVH were obtained by PCR and connected with pET-22b(+). Products were named pET-scFv7E3 and pET-scFvCD62P. The 7E3VL-CD62PVH and CD62PVL-7E3VH were obtained by using overlap PCR and were ligated to pET-22b(+). The products were named pET-ED1 and pET-ED2. The PCR was performed by taking pET-ED2 as a template to obtain the complete operon gene and was ligated to pET-ED1. The product was named pET-7ECD.

**Results:** The identification by restriction endonuclease cleavage and DNA sequencing confirmed that the construction of these expression plasmids was successful. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot confirmed that these plasmids expressed correctly.

**Conclusions:** The expression plasmids pET-scFv7E3, pET-scFvCD62P, and pET-7ECD were constructed and expressed successfully, and laid a good foundation for further research on target-oriented thrombolytic agents.

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Thrombosis is a common clinical disease. Anticoagulation, thrombolysis, and operative treatment may be given to patients suffering from thrombosis. Patients need to take anticoagulation drugs over a long period, they often find it hard to persist in taking drugs and the drugs themselves have many side effects.<sup>1</sup> Therefore, to use an accurate and effective thrombolytic agent that can dissolve thrombosis quickly is an ideal treatment of thrombosis. Nowadays people have more knowledge of the mechanism of thrombosis and its dissolution, breakthroughs have been made and the thrombosis can be prevented and treated to a certain extent.<sup>2</sup> However, myocardial infarction and peripheral arterial embolism and other such diseases may still bring about a high incidence of mortality and injury. Thrombolytic agents are generally divided into 3 generations according to their history of development, the first and second generations lack specificity and will cause bleeding and allergic reaction.<sup>3</sup> The third generation is a result of reforming the first and second generations, including mutant, chimera and thrombus-targeted agents, and so on, but the problems of intracranial hemorrhage and local bleeding reactions are not solved completely.<sup>4</sup> The elimination and metabolism of biological preparation in circulation are not clear, so the safety cannot be ensured.<sup>5</sup> The research on targeting thrombolytics has become a hot issue since Bode et al<sup>6</sup> connected anti-fibrin's monoclonal antibody (mAb) 59D8 with urokinase (UK) by covalent bonding. Targeted thrombolytics are a new type of agent with high-specific avidity to the thrombus because the specific antibodies of the thrombus are connected to the thrombolytic by chemical or biological methods, it has higher efficiency and may reduce reaction to non-target tissues.<sup>7</sup> Current research on targeted thrombolytics implement the target-orientation function of the agent by using the high-specific avidity between mAb and the corresponding antigen. The mAb can be crossly linked to molecules of the thrombolytic, or the mAb can be used as a targeting thrombolytic directly.<sup>8</sup> In the process of thrombosis, blood platelets form persistent platelet aggregation. At the same time, the fibrin consolidates the aggregation internally and peripherally.<sup>9</sup> Cross-linked fibrin and activated platelets are main parts of thrombus and play important roles in the formation, development, and enlargement of thrombus.<sup>10</sup> Platelet glycoprotein GPIIb-IIIa complex (GPIIb-IIIa) is a kind of membrane-bound protein, it will not bind any ligand in a quiescent state. In the process of thrombosis,

blood platelets are activated, which makes GPIIb-IIIa receptors bind with fibrinogen or other adhesive GP, leading to platelet aggregation.<sup>11</sup> P selectin (CD62P) is a member of the selectin family and exists inside the thrombocytic alpha-granule. After endothelial cells are stimulated, CD62P would transfer from the Weibel Palade corpuscula to cell surfaces. When the platelets are activated, CD62P will blend with plasmalemmas on the surfaces of platelets, and express on the platelet membranes. Few CD62P exists on the quiescent platelets.<sup>12</sup> Therefore, CD62P has become one of the molecular markers of platelet activation.<sup>13</sup> The CD62P also can influence the aggregation of platelets, and is a marker of platelet activation.<sup>14</sup> Merten et al<sup>15</sup> demonstrated that CD62P interaction with a ligand, stabilizes initial GPIIb-IIIa-fibrinogen interactions, allowing the formation of large stable platelet aggregates. At present, the clinical application of mAb Abciximab (c7E3) has been approved by the FDA. In adjunctive therapy of acute coronary syndrome and percutaneous coronary intervention (PCI), Abciximab can effectively reduce the occurrence of cardiovascular ischemia caused by platelets.<sup>16</sup> Jiang et al<sup>17</sup> blended SZ51, the mAb of CD62P with low molecular weight pro-urokinase (scu-PA-32K) or pro-urokinase (scu-PA) and obtained chimeric molecules. In vitro analysis indicates that the antibody avidity of the chimeric is 67% of that of mouse original mAb and the in vitro efficiency is 4.1-8.4 times that of uPA.<sup>17</sup> However, mAb has a large molecular weight. Although the single chain variable fragment (scFv) could be applied, only one site could be used for antigen binding and therefore has limited avidity. In this paper, expression plasmids of diabody and 2 kinds of scFv were constructed successfully by re-constructing mAb 7E3 of anti-GPIIb-IIIa and mAb WAPS12.2 of anti-CD62P, the plasmids expressed correctly. Theoretically, the diabody can identify and bind 2 specific antigens, namely, GPIIb-IIIa and CD62P simultaneously and therefore is remarkably superior in improving specificity and functional avidity to thrombus, which lay a good foundation for further research on target-oriented thrombolytics.

**Methods.** The experiment was performed under ethical approval by the Ethical Committee of Wuhan University. This study was carried out at the Laboratory of Molecular Biology, Hunan Yuntai Biological Technology Co. Ltd, China from September 2007 to May 2008. Hybridoma cell lines 7E3 (HB8832) and WAPS12.2 (HB299) were purchased from ATCC, USA. Competent cells JM109 and DE3 were donated by the Institute of Virology, Wuhan University. Cloning vector pGEMT was purchased from Promega Company, USA. Expression plasmid pET-22b(+) was purchased

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from Novagen Company, USA. The Rneasy mini kit and gel extraction kit were purchased from Qiagen Company, USA. The DNA polymerase and reverse transcription kit access (RT-PCR) were purchased from Promega Company, USA. The DNA Marker DL2000, 15000 and Protein Marker were purchased from TaKaRa Company, Japan. The DNA ligase and all kinds of restriction enzymes were purchased from New England Biolabs Company, USA. Mouse anti-His-tag antibody was purchased from Pharmacia Company, USA. Goat-anti-mouse IgG marked by FITC was purchased from Zhongshan Biological Company, China. Other reagents are domestically produced biochemical analytical reagents. The synthesis of primers and gene sequencing were finished with the assistance of Hunan Yuntai Biological Technology Co. Ltd, China. The necessary primers are shown in Table 1. Regular molecular biological operations such as enzyme digestion, connection, and transformation of DNA were carried out according to relevant literatures.<sup>18</sup> Total RNA of monoclonal antibody 7E3 cells and WAPS12.2 cells were obtained using Rneasy Mini Kit, the 7E3 cells, and WAPS12.2 cells. Taking the total RNA as the template, RT-PCR were carried out using reverse transcription kit Access RT-PCR to obtain the genes of variable regions of the light and heavy chains of 7E3 and anti-CD62P. Since the gene sequence of monoclonal antibodies were not clear, synthesizing of multi-pairs of degenerate primers was required and needed to be tested separately. The obtained genes were named 7E3VL, 7E3VH, CD62PVL and CD62PVH. After RT-PCR, agarose gel electrophoresis was carried out. The target genes were retrieved and purified using the QI Aquick Extraction Kits, then were ligated with pGEMT vector. The ligated product was transformed to competent cell JM109 and the white clones were selected to DNA sequencing. Taking the cloning vectors that had been demonstrated to have correct inserting sequences as templates, PCR was carried out to obtain 7E3VL, 7E3VH, CD62PVL, and CD62PVH. After separation and purification, 7E3VL and 7E3VH were put into one PCR tube. Because there was a part of homologous sequence in the middle of the 2 fragments, mixed fragment 7E3VL-7E3VH was obtained by adding corresponding primers to perform PCR. Mixed fragment CD62PVL-CD62PVH was obtained by the same method. The fragments were digested by BamHI and XhoI, then were connected with expression vector pET-22b(+) that also had been digested by BamHI and XhoI. The products were transformed to competent cell JM109, and white clones were selected to be identified by enzyme digestion and DNA sequencing, then were named pET-scFv7E3 and pET-scFvCD62P. The DNA fragments 7E3VL and CD62PVH were ligated by using

overlap PCR and taking G4S as connecting peptide,<sup>19</sup> fragment 7E3VL-CD62PVH was obtained. Fragment CD62PVL-7E3VH was obtained by the same method. Fragments 7E3VL-CD62PVH and CD62PVL-7E3VH were digested by BamHI and XhoI, then were ligated to the expression vector pET-22b(+) that had been digested by BamHI and XhoI. The products were transformed to competent cell JM109 and the white clones were selected to be identified by enzyme digestion and DNA sequencing. The new expression plasmids were named pET-ED1 and pET-ED2. The expression plasmids of heterozygous variable fragment genes were obtained. The PCR was performed by taking pET-ED2 as a template to obtain the complete operon gene including T7 promoter, complete open reading frame of insertion gene and T7 terminator. Expression plasmid pET-ED1 was digested by SphI and was ligated to the operon gene that had already been digested by SphI. The product was transformed to competent cell JM109 and white clones were selected to be identified by enzyme digestion and DNA sequencing. The plasmid was named pET-7ECD. The procedure of the construction of recombinant plasmid pET-7ECD was described in Figure 1. The expression plasmids pET-scFv7E3, pET-scFvCD62P, and pET-7ECD were transformed to competent cells BL21 (DE3). A single colony was selected and inoculated in 1L of Luria-bertani culture solution containing ampicillin (100mg/L). The colony was shaken and cultured in the rocking bed until the A600 was 10. The IPTG was added for induction. The mixture was continued to be shaken and cultured at 37°C. Fifty mL of bacteria splitting liquid was used to

**Table 1** - The primers of polymerase chain reaction.

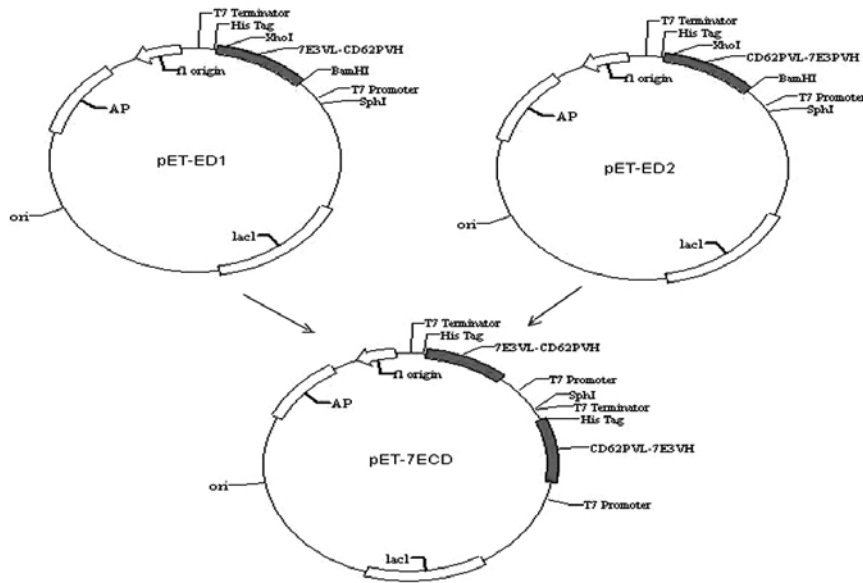
P	Primers	Restriction enzyme site
P1	gggtggtgatccsargtnmagctgsagsagtc	BamHI
P2	gggtggtgatccsargtnmagctgsagsagtcwgg	BamHI
P3	gggtggtgatccsaggtsmarctgcagsagtct	BamHI
P4	gggtggtgatccaggtsmarctgcagsagtcwgg	BamHI
P5	gggtggtgaattcsargtnmagctgsagsagtc	EcoRI
P6	gggtggtgaattcsargtnmagctgsagsagtcwgg	EcoRI
P7	gggtggtgaattcsaggtsmarctgcagsagtct	EcoRI
P8	gggtggtgaattcsaggtsmarctgcagsagtcwgg	EcoRI
P9	agaaccaccgccacatagacagatgggggtgctgttttggc	
P10	gggtggtggttctgayattggtmtsacmcarwctmac	
P11	gggtggtctcagggatacagttggtgcagcatc	XhoI
P12	gggtggtgaactggatacagttggtgcagcatc	HindIII

The underlined is the site of restriction enzyme. Among which, from p1 to p8 and p10 contain the degenerate primers.

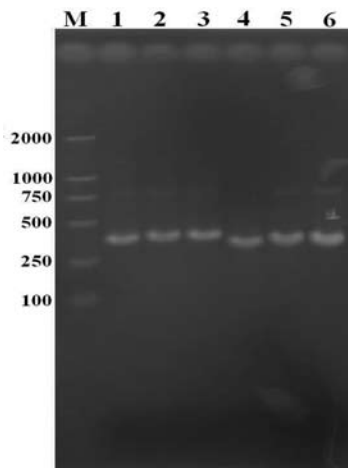
suspend the bacteria, the supernatant was centrifuged and collected to obtain the crude extract of expression product. Samples at each step were analyzed and identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blot.

**Results.** The multiple pairs of degenerate primers were synthesized, and RT-PCR was performed by taking the total RNA of mAb 7E3 cells and WAPS12.2 cells as the template and using reverse transcription kit Access RT-PCR System to obtain variable region genes of light and heavy chains of 7E3 and anti-CD62P.

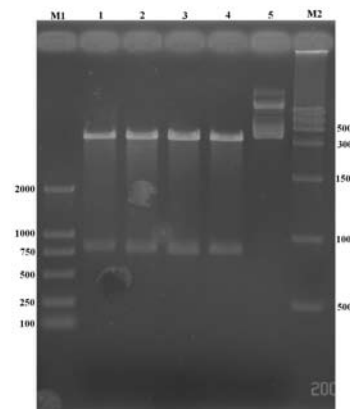
The 7E3VH and 7E3VL were around 350bp, and the CD62PVH and CD62PVL were around 380bp (Figure 2). Expression plasmids pET-scFv7E3 and pET-scFvCD62P were digested by BamHI and XhoI, 1.2% TAE agarose gel electrophoresis was carried out. The result is shown in Figure 3. The upper line of the first path is the expression plasmid pET-22b(+), which had been digested by BamHI and XhoI, and the size of it is around 5000bp. The lower line of the first path is the fragment CD62PVL-CD62PVH, the size of it was around 760bp. The lower line of the third path is the fragment 7E3VL-7E3VH, the size of it is around



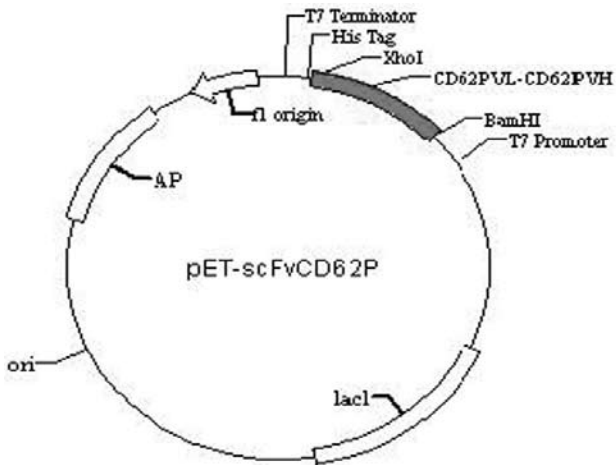
**Figure 1** - Construction of expression plasmid pET-7ECD.



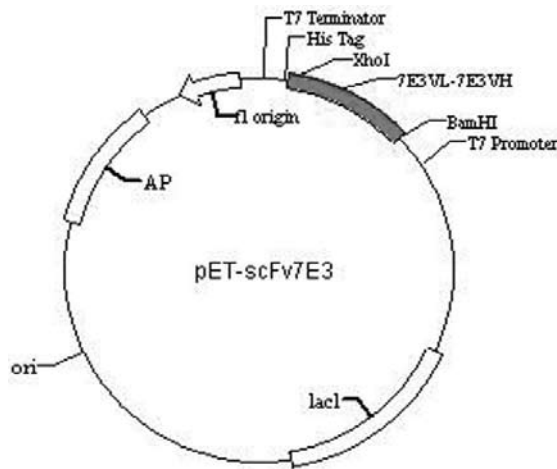
**Figure 2** - Age of target gene. Lane M - DNA marker DL2000. Lane 1: CD62PVL. Lane 2: CD62PVH. Lane 3: CD62PVH. Lane 4: 7E3VL. Lane 5: 7E3VH. Lane 6: 7E3VH. 7E3VH and 7E3VL were approximately 350bp, CD62PVH and CD62PVL were approximately 380bp.



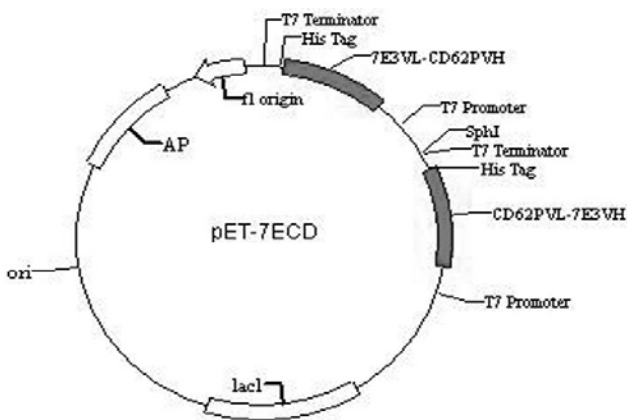
**Figure 3** - Expression plasmid digested by BamHI and XhoI. Lane M1: DNA Marker DL2000. Lane 1: pET-ScFvCD62P, upper line is pET-22b(+) digested by BamHI and XhoI, approximately 5000bp, lower line is CD62PVL-CD62PVH, approximately 760bp. Lane 2: pET-ED1. Lane 3: pET-ScFv7E3, lower line is 7E3VL-7E3VH, approximately 700bp. Lane 4: pET-ED2. Lane 5: pET-ScFvCD62P (uncut). Lane M2: DNA Marker DL15000.



**Figure 4** - Structure of expression plasmid pET-single chain variable fragment (scFv)7E3.

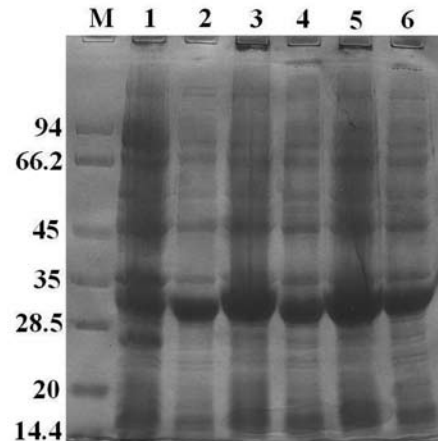


**Figure 5** - Structure of expression plasmid pET-single chain variable fragment (scFv) CD62P.

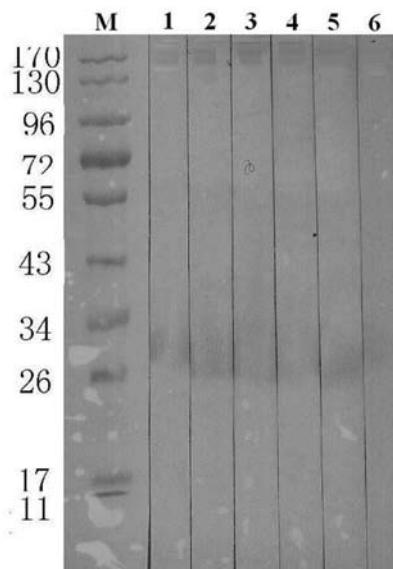


**Figure 6** - Structure of expression plasmid pET-7ECD.

700bp. Their nucleotide sequence was identified by DNA sequencing. The structures of the expressions plasmids are shown in Figures 4 and 5. To avoid intra strand pairing, the carboxyl terminal of 7E3VL gene and the amido terminal of the CD62PVH gene were ligated by short peptide G4S and the carboxyl terminal of CD62PVL gene, and the amido terminal of 7E3VH gene were also ligated by the short peptide G4S, then they were conjugated with the expression vectors. Thus, the heterotic Fv gene was obtained. The expression plasmid



**Figure 7** - Sodium dodecyl sulfate polyacrylamide gel electrophoresis of single chain variable fragment (scFv) and Diabody Lane M: protein Maker. Lane 1: double expression of pET-ED2 and other insertion element. Lane 2: double expression of diabody, the heterozygous protein almost have same molecular weight (31.1KD and 31.4KD), so they are shown as one line. Lane 3: expression of pET-ED1. Lane 4: expression of pET-ED2. Lane 5: expression of pET-scFv 7E3, approximately 31.1KD. Lane 6: expression of pET-ScFvCD62P, approximately 31.2KD.



**Figure 8** - Western Blot of single chain variable fragment (scFv) and diabody.

of diabody pET-7ECD was constructed by PCR, SphI digestion, and ligation reaction (Figure 6). Its nucleotide sequence was identified by DNA sequencing. In the expression plasmid, each of the 2 heterotic genes has a set of T7 promoter and terminator without mutual influence. Under the condition of hypophosphate, the secreting expression was implemented, and the chimeric scFv was formed in the periplasmic space of the bacteria. The expression plasmids pET-scFv7E3, pET-scFvCD62P, and pET-7ECD were transformed to BL21 (DE3), then were induced to express by hypophosphate. The secretion of scFv and diabody was guided by signal peptide. Then the product entered the periplasmic space of the bacteria. The expression product of the periplasmic space of bacteria was extracted using hypertonic solution. The expression products of pET-scFv7E3 and pET-scFvCD62P were analyzed by 12% SDS-PAGE, and 2 protein lines with respective molecular weights of 31.1KD and 31.2KD were shown (Figure 7, the fifth and sixth paths). The expression product of pET-7ECD was analyzed by 12% SDS-PAGE. Because the heterozygous protein of 7E3VL-CD62PVH and CD62PVL-7E3VH have almost the same molecular weight (31.1KD and 31.4KD), the 2 proteins were shown as one line on the electrophoretogram (Figure 7, the second path). The western blot result showed that anti-His tag antibody could hybridize with the protein of 2 single-chain antibodies and diabody (Figure 8, path 1, 2, and 4).

**Discussion.** Since Kohler and Milstein created lymphocyte hybridoma technique in 1975,<sup>20</sup> the application of the mAb technique has been widely used on basic research and clinical diagnosis. However, the application of mAb to disease treatment does not achieve satisfactory effects. This is mainly because most mAbs applied are taken from mice and may easily produce human antimouse antibody. At the same time, mAbs usually have bigger molecular weights, so it is difficult to penetrate the target tissue and then the therapeutic effect is weaker.<sup>21</sup> The single chain variable fragment is a recombinant protein, formed by immune globulin's light and heavy chains of the variable region connecting through a fragment of connecting peptide. It is the minimal antibody fragment with complete antigen binding site, with a size of one sixth of a complete antibody and a molecular weight of 27KD.<sup>22</sup> Due to its smaller size and lower immunogenicity, the reaction of anti-heterologous protein will not happen when it is used in vivo, and it may have easy access to the microcirculation around the target antigen. Besides, because of no Fc fragment, it will not bind nontarget cells having Fc receptors.<sup>23</sup> However, scFv only has one antigen binding site, and therefore has limited avidity to

target the tissue.<sup>24</sup> Also, the mutual effort between VL and VH inside the scFv molecule is not strong enough to counteract intermolecular force, so the dimer and polymer are easy to form.<sup>25</sup> Holliger put forward the concept of diabody in 1993,<sup>26</sup> the basis of diabody is single chain variable fragment. The genes of heavy-chain and light-chains of variable regions of 2 antibodies are cross-linked to form a hybridizational single chain variable fragment gene. After expression, the double chains of 2 single-chain antibodies automatically lap over each other to form double or double-specific antibody fragments that can identify and bind 2 kinds of specific antigens,<sup>27</sup> having obvious advantage in improving specificity and functional avidity to thrombus. The antibody fragment is a double-functional antibody with the minimal molecular weight, which is one third of that of IgG, having lower immunogenicity and stronger penetrating power.<sup>28</sup> In this study, because the sequences of target genes were not known, in order to amplify homologous gene sequence, multi-pairs of degenerate primers were designed. By using the monoclonal antibody 7E3 of anti-GPIIb-IIIa and the monoclonal antibody WAPS12.2 of anti-CD62P, the genes of light-chain and heavy-chain of variable region of 7E3 and anti-CD62P were obtained. For obtaining high-efficient expression of genes and considering the property of using prokaryotic cells to express eukaryotic protein, we successfully constructed the expression plasmids of pET-scFv7E3, pET-scFvCD62P, and pET-7ECD. Gene sequencing and identification by enzyme digestion proved that the construction is successful. In the newly expressed plasmid of the diabody, each of the 2 heterotic genes has a set of T7 promoter and terminator without mutual influence. After eliminating the repression function of lac by IPTG, the 2 heterotic genes of the diabody realize secretional expression in BL21 (DE3), and then form the chimeric scFv in the periplasmic space of bacteria, forming a double-specific antibody fragment. Both SDS-PAGE and Western blot analysis demonstrated that both expression plasmids of single chain variable fragment and diabody have correct expressions. In this study, theoretically, the new diabody may simultaneously identify and bind 2 specific antigens, namely, GPIIb-IIIa and CD62P, having the advantages of smaller molecular weight, higher antigen binding power, and comparatively lower immunogenicity, and therefore is obviously superior in improving specificity and functional avidity to thrombus. The diabody itself can inhibit platelet aggregation and has the function of anti-activated platelets, if it is conjugated with the thrombolytic agent, an ideal target-oriented thrombolytic drug would be produced. In consideration of the capacity of the protein yield, determination of binding activity and the affinity constant of recombinant

protein to target antigen was not carried out, and the experimental study on coupling the recombinant protein with a thrombolytic drug was not carried out, either. Whether the recombinant protein can be applied in vivo still needs further experimental demonstration. The next stage of the experiment will search for a more suitable expression vector to make the expression of the diabody more effective, the binding activity and affinity constant of the recombinant protein to activated platelet will be determined, and the recombinant protein will be conjugated with a thrombolytic drug to discuss the target-oriented thrombolysis in vitro. The results of this study preliminarily demonstrated that the expression plasmid of anti-CD62P/anti-GPIIb-IIIa diabody could be constructed by genetic engineering technology, with correct expression, which lays a good foundation for further research on target-oriented thrombolytic drugs.

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