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Full Length Research Paper

Expression, purification and characterization of recombinant targeting bifunctional hirudin in *Pichia pastoris*

Zongli Hu, Ning Zhang, Feng Gu, Yong Li, Xiaojie Deng and Guoping Chen*

Key Laboratory of Biorheological Science and Technology (Chongqing University), Ministry of Education, Bioengineering College, Chongqing University, Chongqing 400044, People's Republic of China.

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A recombinant targeting bifunctional hirudin was expressed in the yeast *Pichia pastoris*. In order to decrease the side effects of hirudin and increase its activity to prevent arterial thrombus, we fused a factor Xa (FXa) recognition sequence into N' of hirudin, while maintaining the activity of natural hirudin. In addition, an Arg-Gly-Asp (RGD) sequence was fused into appropriate genetic locus of hirudin. Furthermore, we added a 9 × His - Tag to make its separation and purification conveniently. The recombination hirudin gene was successfully cloned and ligated into the *P. pastoris* vector pPIC9K to form an expression vector, which was transferred into *P. pastoris* GS115. A transformant strain was selected and expressed efficiently in suitable conditions. Then, the fusion protein was purified by affinity chromatography. Through anti-thrombin activity analysis and anti-platelet aggregation activity analysis, we found that the anti-thrombin activity of the fusion protein did not change comparing with the natural hirudin, whereas its anti-platelet capability was enhanced.

Key words: Targeting, bifunctional, hirudin, recombinant, anticoagulant, *Pichia pastoris.*

INTRODUCTION

Cardiocerebral vascular diseases lead to about 17 million deaths every year around the world. Currently, scientists are focusing more than ever on discovering effective anti-thrombin drugs (Breddin, 2002). Hirudin is a potent thrombin inhibitor, which has a molecular weight of 7,000 Da secreted from salivary glands of *Hirudo medicinalis*. Thrombin converts fibrinogen to fibrin, which is the final step in the process of hemostatic clot formation. Hirudin binds to the active site of thrombin, preventing thrombin's interaction with fibrinogen, thereby blocks subsequent blood coagulation (Markwardt, 1989; Rosenfeld et al., 1996). As an effective drug to prevent thrombus, hirudin has various advantages, such as the stable and sustained anti-thrombus activity, the specific thrombin inhibition activity, rare side effects, teeny antigenicity, little toxicity for its low molecular weight and so on. However, like heparin, hirudin exhibits significant bleeding side effects. Even worse, there are rare antagonists in bleeding induced by hirudin. Furthermore, hirudin is only effective in phlebothrombosis but not in arteriothromobosis (Breddin, 2002).

In order to overcome its side effects and to increase its activity to prevent arteriothromobosis, we fused a FXa recognition sequence into N-terminal' of hirudin to minimize the effect of bleeding through elevating of its specific targeting while maintaining the activity of natural hirudin (Harker et al., 1997; Mann, 1994; Niu et al., 2006). In addition, we fused an Arg-Gly-Asp (RGD) sequence into appropriate genetic locus of hirudin to elevate its activity of platelet aggregation inhibition, which would greatly increase its activity of preventing arterial thrombus formation (Bennett, 2001; Smith et al., 1995; Zhang et al., 2006; Zhou et al., 2001; Zokai et al., 2001). Furthermore, we added a 9×His-Tag to make its separation and purification conveniently, which makes the large amount of fusion hirudin protein production available. The recombination hirudin gene was successfully synthesized and ligated into the *Pichia*

*Corresponding author. E-mail: chenguoping@cqu.edu.cn. Tel: 0086 23 65112674. Fax: 0086 23 65102507.

Table 1. Sequences of primers.

Primer	Sequence
Hv 1	5'-AAGGATCCGATGATGATGATAAGATTGAGGGTCGTGTTGTTTACACTGAT-3'
Hv 2	5'-ACCCTCGCAAAGGCAAAGGTTTTGACCAGACTCGGTGCAATCAGTGTAAACAAC-3'
Hv 3	5'-TGCCTTTGCGAGGGTTCCAACGTTTGCGGTCAAGGTAACAAGTGCATTCTTGGT-3'
Hv 4	5'-AACGCATTGGTTCTTATCACCACGACCAAGAATGCACTT-3'
Hv 5	5'-AAGAACCAATGCGTTACTGGTGAAGGTACTCCTAAGCCTCAATCTCATAACGATGGTG-3'
Hv 6	5'-TTGGATCCTTATTGAAGGTAATCCTCAGGAATTTCTTCGAAGTCACCATCGTTATGAG-3'
A	5'-CGGAATTCCACCACCACCACCACCACCACCACCACCACGGATCCGATGATGATGATAA-3'
B	5'-TTGCGGCCGCTTATTGAAGGTAATCCTCAG-3'
pPIC9K 1	5'-TACTATTGCCAGCATTGCTGC-3'
pPIC9K 2	5'- GGCAAATGGCATTCTGACATC-3'

pastoris vector pPIC9K to form an expression vector, which was transferred into *P. pastoris* GS115 successfully as evidence by the functional assay with anti-thrombin and anti-platelet aggregation activity analysis.

MATERIALS AND METHODS

Enzymes, cell strain and plasmid

Escherichia coli JM 109 strains, the plasmid vector pMD18-T, restrictive endonucleases, *Taq* DNA polymerase, T4 DNA ligase, were purchased from TaKaRa biotechnology. The plasmid expression vector pPIC9K and *P. pastoris* expression host strain GS115 were purchased from invitrogen. The standard mini plasmid Prep kit and the DNA gel extraction kit were purchased from Bioteke. BCA protein assay kit was purchased from Pierce. The natural hirudin, the fibrinogen and the thrombin were purchased from sigma. The enterokinase (EK) was purchased from Kerun biopharmaceutical R&D Co. Ltd (Chongqing, China). The factor Xa (FXa) was purchased from Promega.

The design of primers

According to the requirement of experiments, 6 primers, Hv1, Hv2, Hv3, Hv4, Hv5 and Hv6, were designed for the combination of the DNA sequence of a fusion protein, named as r-HV (fusion hirudin linked with FXa recognition sequence and RGD sequence). Primer A and B were designed for ligating the gene fragment of the recombinant protein r-HV into the expression vector pPIC9K and pPIC9K1 and pPIC9K2 were designed for the identification of the positive colonies. Sequences of these primers are shown in the Table 1.

Synthesis of the fusion protein gene

The DNA sequence of our fusion protein was achieved by 3 rounds of PCR (Figure 1). The first round PCR reaction systems: 10 × PCR buffer 5.0 ul, $MgCl_2$ (25 mM) 3.0 ul, dNTPs mixture (10 mM) 1.0 ul, *rTaq* (5 U/ul) 0.5 ul, Primer Hv1/ Hv3/ Hv5 (10 uM) 1.0 ul, Primer Hv2/ Hv4/ Hv6 (10 uM) 1.0 ul, add ddH_2O to 50.0 ul. PCR reaction conditions: 94℃ 5min 1cycle; 94℃ 30s, 55℃ 30s, 72℃ 30s 35 cycles; 72℃ 10min.

After the first round PCR, 3 DNA fragments including product 1, product 2 and product 3 can be obtained respectively. Then with primers Hv1 and Hv4, the second round PCR was carried out and yielded product A by using the product 1 and product 2 as templates. At the same time, another PCR product B was amplified by using the product 2 and product 3 as templates, with primers Hv3 and Hv6. Finally, the third round PCR was carried out and the fusion protein gene r-HV was acquired with primers Hv1 and Hv6 and the product A and product B as templates. The second and third round PCR reaction conditions are the same as the first round PCR. Then, the r-HV was purified and sub-cloned into pMD18-T to yield recombinant plasmid pMD18-T-r-HV.

Construction of expression vector pPIC9K-r-HV

The r-HV gene fragment was amplified from plasmid pMD18-T-r-HV with primer A and primer B. Primer A contains an *Eco*RI site and a 9×His-Tag sequence. Primer B contains a *Not*I site to facilitate sub-clone. Digested with *Eco*RI and *Not*I, the above PCR product was ligated into pPIC9K to construct the expression vector pPIC9K-r-HV. The recombinant was identified by restriction enzyme analysis and sequencing (Invitrogen in Shanghai).

Expression and purification of fusion protein r-HV

The pPIC9K-r-HV plasmid was linearized with *Sal*I and was electroporated into *P. pastoris* strain GS115 with the condition of 1500 V, 25 μF and 400 Ω, with 1 mL pre-cooled 1 mol/L D-sorbitol. The cells were plated onto YPD medium(1 yeast extract, 2 peptone, 2 dextrose, 2% agar) containing 0, 1.0, and 2.0 mg/ml G418. The Mut+ and Muts phenotypes of the transformants were evaluated by spotting them on the MD plates (1.34% YNB, 2% dextrose, 4 $\times 10^{-5}$% D-biotin, 1.5% agar) and the MM plates (1.34% YNB, 0.5% methanol, 4 $\times 10^{-5}$% D-biotin, 1.5% agar). The positive transformants harboring r-HV were identified by PCR and grown overnight in 5 ml BMGY medium (1% yeast extract, 2% tryptone, 100 mM phosphate potassium buffer, 1.34% YNB, 4 × 10^{-5}% D-biotin and 1% glycerol) at 30℃ with shaking. The cultures were centrifuged and the cell pellets were resuspended in 1:10 the original volume in MM medium (1.34% YNB, 0.5% methanol, 4 × 10^{-5}% D-biotin). After 2 days culturing under the conditions mentioned above, the expression of r-HV protein was induced with daily additions of 1.0% v/v methanol. Then the harvested supernatant was centrifuged at 4,000 g for 20 min and the interested protein was purified by affinity chromatography and analyzed by SDS–PAGE gel.

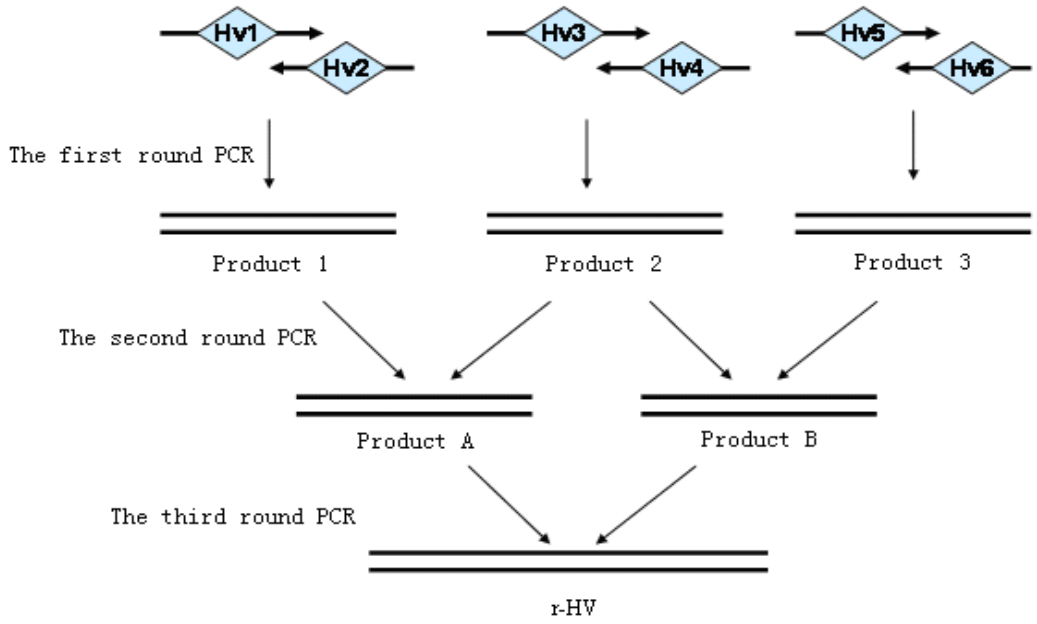

Figure 1. PCR procedures.

Anti-thrombin activity analysis

The anti-thrombin activity of the recombinant protein was evaluated with anti-thrombin titration method as previously described (Markwardt et al., 1970; Markwardt, 1994). Different concentrations of samples were prepared. Then, direct titrations with thrombin were performed respectively, in a 37°C constant temperature container.

Anti-platelet aggregation activity analysis

The anti-platelet aggregation activity was assayed by turbidity method as described previously (Imura et al., 1992). Venous blood was collected from healthy human donors, who didn't take any drug 2 weeks before the donation. Blood samples were centrifuged at 950 rpm for 10 min or 2,000 rpm for 10 min at room temperature to obtain platelet rich plasma (PRP) or platelet poor plasma (PPP) respectively. Platelet aggregation was measured with multi-functional intelligence blood condense-meter of TYXN-96 series (Shanghai Research Institute of General Electronics and Machinery). The r-HV was added into the cuvettes with PRP solution to with final concentration as 0.001 and 0.00015 mg/ml respectively. After 15 min incubation at room temperature, 5 ul ADP (final concentration: 5 uM) was added into cuvettes to determine the maximal platelet aggregation.

RESULTS

Synthesis of the fusion protein gene r-HV

In order to minimize the side effects of hirudin and to increase its activity to prevent arterial thrombus formation, we designed 6 primers, Hv1, Hv2, Hv3, Hv4, Hv5 and Hv6, for the combination of the nucleotide sequence of the fusion protein r-HV, which has specific targeting and anti-platelet aggregation activities without affecting natural hirudin activity. Through 3 rounds of PCR, the DNA fragment of the fusion protein r-HV was obtained. After subcloning the fragment and analyzing the nucleotide sequence of the clone, one DNA fragment was obtained. The nucleotide sequence and the deduced amino acid sequence of the target gene were shown in Figure 2.

Construction of expression vector pPIC9K-r-HV

A 9×His sequence was fused into the 5' terminal of *r-HV* gene by PCR, which would make the purification of r-HV protein conveniently. Digested with *Eco*RI/*Not*I, the fusion r-HV fragment was sub-cloned into the pPIC9K plasmid. Thus, the fusion protein expression cassette contains 9×His-tag, an EK recognition site, a FXa recognition site and the hirudin contained RGD sequence in turn from the N terminal to C terminal (Figure 3).

Induced expression of r-HV in *P. pastoris*

After transferring the expression plasmid into *P. pastoris* strain GS115 by electroporation, 5 positive transformants were selected and induced by methanol. After 4 days culture, 1 ml expression supernatant was centrifuged and analyzed by SDS–PAGE gel. A maximal yield of foreign protein was attained in the transformant 2 (lane 2) (Figure 4) with 2 target bands, which might be resulted from the degradation of the recombinant protein because of the long inducing duration.

```
GGATCC GAT GAT GAT GAT AAG ATT GAG GGT CGT GTT GTT TAC ACT   45
       D   D   D   D   K   I   E   G   R   V   V   Y   T    13
GAT TGC ACC GAG TCT GGT CAA AAC CTT TGC CTT TGC GAG GGT TCC   90
 D   C   T   E   S   G   Q   N   L   C   L   C   E   G   S   28
AAC GTT TGC GGT CAA GGT AAC AAG TGC ATT CTT GGT CGT GGT GAT  135
 N   V   C   G   Q   G   N   K   C   I   L   G   R   G   D   43
AAG AAC CAA TGC GTT ACT GGT GAA GGT ACT CCT AAG CCT CAA TCT  180
 K   N   Q   C   V   T   G   E   G   T   P   K   P   Q   S   58
CAT AAC GAT GGT GAC TTC GAA GAA ATT CCT GAG GAT TAC CTT CAA  225
 H   N   D   G   D   F   E   E   I   P   E   D   Y   L   Q   73
TAA GGATCC                                                  234
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Figure 2. Nucleotide and deduced amino acid sequences of r-HV gene.

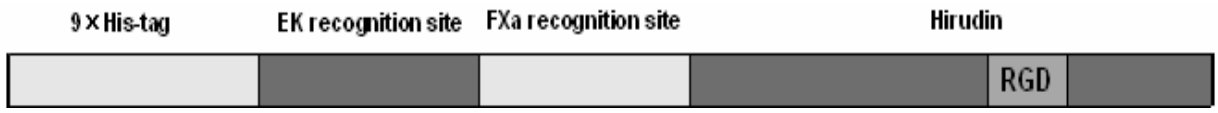

Figure 3. The fusion protein expression cassette.

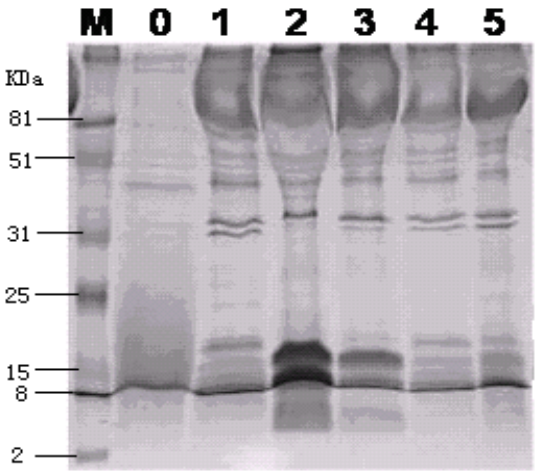

Figure 4. SDS-PAGE result of the induced products. Lanes 1-5: culture supernatant of corresponding transformants 1-5. Lane 0: empty vector pPIC9K induced 4 days. Lane M: molecular mass marker.

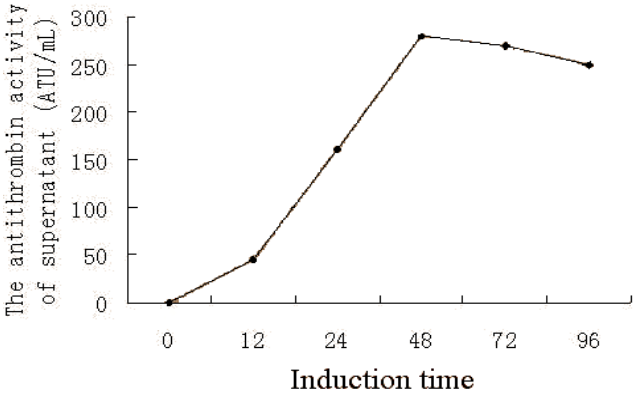

Figure 5. The anti-thrombin activity analysis of products after methanol inducement. Results represent the mean values from three independent experiments.

Anti-thrombin activity analysis of the expression supernatant

To confirm the expression efficiency of the target protein from the transformant 2 and to determine the optimal induction time, samples of the expression medium supernatants of the transformant 2, taken at various time points, were treated with ultrafiltration centrifugalization and digested by factor Xa (FXa), then the anti-thrombin activity at different induction time was measured with anti-thrombin titration by the method of Markwardt et al. (1970, 1994). During induction, the anti-thrombin activity of the recombinant protein increased to 280 ATU/ml in a time dependent manner within 48 h (Figure 5) which implies that the optimized inducing time of the transformants was 48 h. Longer induction might result in the lose of anti-thrombin activity of the target protein, as evidence by the result from SDS-PAGE (Figure 4).

Purification and SDS-PAGE analysis of recombinant protein

The recombinant strain was concentrated by 10-fold ultrafiltration centrifugation. Then it was purified through affinity columns with AKTA and HiTrap™ pre-

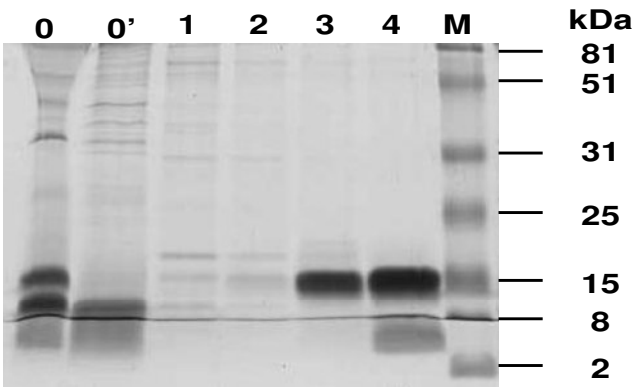

Figure 6. SDS-PAGE analysis of the purified products. Lane 0: total protein without purification. Lane 0': product purified through affinity columns without affinity adsorption. Lane 1-4: eluted products at various time intervals. Lane M: molecular mass marker.

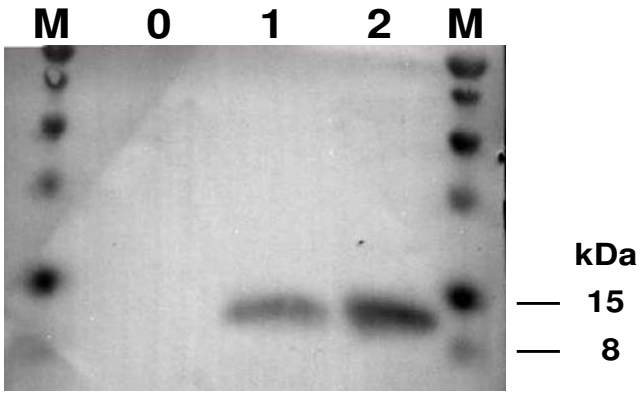

Figure 7. Western-blot identification of the purified products.Lane 1: product purified by ultrafiltration centrifugation and affinity chromatography from expression supernatant after 2 days induction culture. Lane 2: product purified by ultrafiltration centrifugation from expression supernatant after 2 days induction culture. Lane 0: the control group without induction expression. Lane M: molecular mass marker.

packed columns (Pharmacia). The purified recombinant protein was analyzed by SDS–PAGE (Figure 6). A single 15 kDa protein band about 15kDa molecular weight was obtained at propriety proper elution time (lane 3). Because hirudin is a small acidic protein, although the theoretical molecular weight of the recombinant protein should be 9.6 kDa, it may be less negatively charged in SDS-PAGE and shows low mobility, which is might be responsible for lower motility and the molecular weight increase (Bennett, 2001). The BCA protein concentration assay showed that the total protein level was 1.54 mg/ml and the interest r-HV protein level was 23.1 mg/L, which implied higher efficiency than other recombinant hirudin expression systems (Bar-Shavit et al., 1989).

Western blotting analysis of purified recombinant protein

As the target protein contains a 9×His-Tag recognition sequence in its N terminal, we analyzed the purified recombinant protein by western blotting with anti-his antibody (Figure 7). The western blotting showed that the expression of target protein was undetectable before methanol induction (lane 0). A single target protein band was observed in the expression supernatant after ultrafiltration centrifugation and affinity chromatography.

Anti-thrombin activity assays

As the expression protein has an EK and a FXa recognition sequence in its N terminal, EK and FXa have been selected to digest the purified recombinant protein. If digested with EK, one fusion protein with a FXa recognition sequence in its N terminal was obtained. If digested by FXa, another protein without a FXa recognition sequence in its N terminal was obtained. The anti-thrombin activities of proteins, those before and after digestion by EK and FXa, were measured with anti-thrombin titration by the method of Markwardt et al. (1970, 1994). The results were shown in Table 2.

The recombinant protein, which contains a 9×His-Tag, an EK and an FXa recognition sequence in its N terminal, had low anti-thrombin activity, which indicated that the hirudin would lose anti-thrombin activity with long amino acid sequences in its N terminal. The recombinant protein with only an FXa recognition sequence in its N terminal had a partial anti-thrombin activity, which might be attributed to that the FXa recognition sequence has only 4 amino acid residues and could not completely inhibit the anti-thrombin activity. The protein digested by FXa had a similar anti-thrombin activity with natural hirudin.

In Table 2, sample A was the recombinant protein without digestion. Sample B was the protein digested by EK, which was the interest protein. Sample C was the protein digested by FXa, which was the bifunctional hirudin after activating by digested *in vivo* conditions. Sample D was the standard hirudin (0.1 mg /ml).

Anti-platelet aggregative activity assays

The anti-platelet aggregative activity was assayed by turbidity method. The inhibition of platelet aggregation was calculated by the following formula:

The inhibition of platelet aggregation (%) = (platelet aggregation of the control group%-the recombinant protein-induced platelet aggregation %)/platelet aggregation of the control group% ×100% (Table 3).

Table 2. Anti-thrombin activities analysis of fusion proteins.

Assay	A	B	C	D
Anti-thrombin activity (ATU/ml)	280	8040	16760	1190
Specific activity (ATU/mg)	182	5201	10883	11900

Table 3. Anti-platelet aggregation activities analysis of fusion protein

Assay	ADP (5 uM)	Physiological saline	FXa-RGD–HV (0.001 mg/ml)	FXa-RGD–HV (0.00015 mg/ml)
Platelet aggregation PAG(M)	92.7%	94.2%	74.7%	81.1%
Inhibition of platelet aggregation (%)	-	-	20.7%	13.9%

The results indicated that the fusion protein, FXa-RGD–HV, had high anti-platelet aggregative activity. With physiological saline as control, the inhibition of platelet aggregation (%) of FXa-RGD–HV at the following concentrations, 0.001 and 0.00015 mg/ml, were 20.7 and 13.9%.

DISCUSSION

Research in the cardiocerebral vascular diseases has made great progress, especially in the development of protein, peptide, or small molecules to inhibit blood coagulation proteases (Dodt et al., 1984; Seemmuller et al., 1986). Hirudin is a natural potent thrombin inhibitor, which can hold back the formation of fibrins in the coagulation pathway. At present, it has been studied extensively as an anticoagulant and should lead to targeted approaches to anticoagulant therapies (Rosenfeld et al., 1996).

In this paper, we showed the synthesis procedures of a recombinant targeting bifunctional hirudin and an novel expression vector, pPIC9K-r-HV, which can be transferred into *P. pastoris* GS115 and the constructed hirudin was properly expressed and secreted into culture supernatants as a soluble protein. The expression level of the recombinant protein could attain 23.1mg/L level. In the meanwhile, the optimal conditions for expression were determined. The anti-thrombin activity and anti-platelet aggregation activity of recombinant hirudin were analyzed *in vitro*.

Although the expression level of the fusion protein is higher than other recombinant hirudin expression systems (Bar-Shavit et al., 1989), the yield should be promoted further before the clinical use of this recombinant protein. So, we would continue to select multiple copy transformants and optimize culture conditions. In addition, *in vivo* animal experiment study should be performed in the future for confirming the targeting activity and bifunction of the recombinant hirudin further.

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