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Construction, Expression, and Characterization of Recombinant Hirudin in *Escherichia coli*

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Abstract

The mutant gene of HV2-K47 was obtained by polymerase chain reaction–directed mutagenesis and expressed in *Escherichia coli*. Many elements that could affect its expression level were compared. The product was purified to homogeneity via three chromatographic steps—ion exchange, gel filtration, and reverse phase chromatography—on the AKTA Explorer System. The antithrombin activity of HV2-K47 is much higher than that of recombinant HV2. Some properties and expression conditions were investigated systematically, which would be useful for further studies of hirudin and other small proteins.

Index Entries: Antithrombin; expression; purification; recombinant hirudin HV2-K47; secretion.

Introduction

Hirudin is a small protein of 65 or 66 amino acid residues secreted by the salivary glands of the leech *Hirudo medicinalis (1)*. It is the most specific and potent inhibitor of thrombin. The hirudin in leech head mainly comprises HV2, and its bioactivity is higher than the other two variants *(2)*. The native HV2 has sulfate ester on Tyr^{63}, which is important for its bioactivity and is lacking in recombinant HV2 *(3)*. The tripeptide segment Pro_{46}-Lys_{47}-Pro_{48} of some wild-type hirudin variants appears to be important in prompting the hirudin NH_2-terminal active site interaction with thrombin *(4)*. Studies have indicated that the activity of mutant HV2-K47 is increased by 61% in comparison with that of recombinant hirudin *(5)*.

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Previous studies have shown that the high expression of foreign gene in *Escherichia coli* is not easy for small proteins. These problems could be limited by using secretion expression system *(1)*. First, the bacterial signal peptide fused to the N-terminal of the desired protein can be cleaved by appropriate signal peptidase; thus, the secreted products with native terminus will be available and the yield will be high. Second, it is putative that the stability of gene product will be increased for much less protease activity in periplasm than that in cytoplasm *(6)*. Third, the overexpression of foreign proteins may be toxic to the host, and the secretion to periplasm will alleviate this problem and provide more space for transferring the product.

Here we report the cloning, expression, and characterization of recombinant hirudin HV2-K47 in *E. coli*. Some conditions for high level of its expression and purification were also investigated, and some properties of the recombinant were tested.

Materials and Methods

Construction of HV2-K47 Gene and Recombinant Expression Vector

According to the amino acid sequence of hirudin variant2, six pieces of polynucleotides were designed and chemically synthesized. The whole hirudin gene was constructed with these polynucleotides by recursive polymerase chain reaction (PCR) *(7)*. Then the mutant gene of HV2-K47 was obtained by PCR-directed mutagenesis. Primers utilized for amplification were as follows: 5'ACACCGGCCGCAAATGCTATTACTTACACT GATTGTAC3' and 5'AGCAATTGCTTATTGTAAATATTCTTCTGGAAT TTCTTCGAAATCGCCGTTGTTATGGCTTTCAGGCTTCGG TGTACCTTC3'. The signal peptide of protein A was obtained by PCR with the template pLSPAJV6. Primers were as follows: 5'CCCATTAATATG TTGAAAAAAGAAAAACGCCGG3' and 5'TTTGCGTGTTACGCCACC3'. The gene encoding the signal peptide of protein A and mutant HV2-K47 was obtained by recombinant PCR using the previous two PCR products as templates. It was cloned into the expression vector pET21a at *Eco*RI and *Nde*I sites. Recombinant expression vector was named pTS-hir and used to transform *E. coli* BL21(DE3). pLSPAJV6 was provided by Prof. Boliang Li of Academia Sinica of China. pET21a and *E. coli* strain BL21(DE3) were stored by our laboratory.

Expression of HV2-K47 in E. coli

A single recombinant *E. coli* colony was inoculated into 3 mL of Luria-Bertani (LB) medium containing 50 µg of ampicillin/mL and shaken vigorously at 37°C for 8 h. After transfer into 50 mL of LB medium, the cell culture was grown in a shaker at 37°C until OD_{600nm} = 0.6–0.8 (about 2 h). Then it was induced with 0.1 mmol/L of isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 10 h. The culture medium, cell periplasm, and cytoplasm were harvested, respectively, and kept at –20°C for antithrombin activity assay.

Assay of Antithrombin Activity of HV2-K47

The activity of HV2-K47 was measured by its ability to inhibit thrombin-releasing colored 4-nitranilin from the chromogenic substrate Tos-Gly-Pro-Arg-4-NA. Three microliters of thrombin solution (3I U/mL) was diluted with 393 µL of Tris buffer (50 mmol/L of Tris, pH 8.3; 227 mmol/L of NaCl) and mixed with 4 ng of hirudin. The mixture was incubated at 37°C for 5 min and then added to 16 µL of Tos-Gly-Pro-Arg-4-NA solution (2 mmol/L). After mixing, it was incubated at 37°C for 10 min, followed by the addition of 200 µL of acetic acid (33%) to stop the reaction. The absorbance was determined at 405 nm.

Construction of Four Different Signal Peptide–Containing Mutants

The HV2-K47 gene containing different signal peptide sequences (bla, lpp, phoA, proteinA) was constructed by nested PCR. After cleaving with appropriate restriction enzymes, they were cloned into pBV220 vector at *Eco*RI and *Bam*HI sites and introduced into *E. coli* DH5α. Each mutant was inoculated into 3 mL of LB medium and shaken vigorously at 30°C for 2 h, and the culture temperature was raised to 42°C to initiate the induction for 10 h. The cell medium, periplasm, and cytoplasm were harvested, respectively, and used to compare their expression level.

Purification of HV2-K47

Cell culture (500 mL) was centrifuged at 2292 *g* for 5 min. The medium was collected and treated by ultrafiltration. Then it was purified via the AKTA Explorer System (Pharmacia Biotech). The raw extracts were first loaded on a DEAE Sepharose Fast Flow column. The protein was eluted by 2 to 3 vol of 20 mmol/L of Tris-Cl. Fractions containing hirudin were pooled. After ultrafiltration, they were purified on a Superdex 75 column previously equilibrated in 0.15 mol/L of NaCl. Fractions with activities were further purified by a column pepRPC HR 515 (Pharmacia Biotech) used for reverse-phase chromatography and prepacked with porous silica, C_2/C_{18}. The protein of interest was eluted by graduations of acetonitrile containing 0.1% trifluoroacetic acid from 0 to 100%. The purified protein was collected and lyophilized.

Results and Discussion

Optimum Conditions for Expression of HV2-K47

The HV2-K47 recombinant was constructed, and the results of DNA sequencing demonstrated that its sequence was in agreement with that designed previously by ourselves. The expression of HV2-K47 was mainly processed according to the method described above. The effect of inducer concentration on the expression of hirudin was tested. For 10 h after the addition of different concentrations of IPTG, the antithrombin activity was measured, as shown in Table 1.

Table 1
Effect of Inducer Concentration on Recombinant Hirudin Expression at 10 h[a]

IPTG concentration (mmol/L)	0.0	0.1	0.5	1.0
Antithrombin activity (ATU/mL)[b]	Negligible	82	80	81

[a]A concentration of 0.1 mmol/L of IPTG is enough to induce high expression of hirudin.
[b]ATU, antithrombin activity unit.

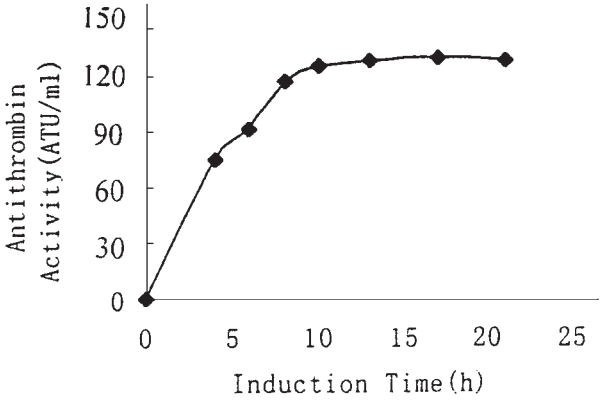

Fig. 1. Time course of recombinant hirudin expression after induction with 0.1 mmol/L of IPTG. The antithrombin activity reached a maximum at 10 h after the addition of IPTG.

The expression of hirudin in pTS-hir/Bl21(DE3) was very sensitive to IPTG. IPTG at very low concentration could induce higher expression of hirudin. Because of the efficiency and specificity, a small quantity of IPTG can cause some expression of T7 RNA polymerase, which is sufficient for the high level of expression of hirudin in pTS-hir/BL21(DE3). Thus, 0.1 mmol/L of IPTG was chosen in following related experiments.

Next, a time course of hirudin expression was done with 0.1 mmol/L of IPTG to optimize the expression of hirudin in pTS-hir/BL21(DE3). The antithrombin activity was measured by colorimetric assay, as shown in Fig. 1. The expression of hirudin in pTS-hir/BL21(DE3) reached a maximum at 10 h after the addition of IPTG. The relationship between cell growth and protein expression is illustrated in Fig. 2. Hirudin expressed highly at 10–12 h after induction. At that time, pH of the medium increased from 7.2 to 8.0–8.5. Because the change in pH was not good for the growth of *E. coli*, we used medium containing phosphate to improve its moderator capacity.

Comparison of Four Different Signal Peptide–Containing Mutants

The antithrombin activities of four different signal peptide–containing mutants are given in Table 2. phoA signal peptide was more suitable for the secretion of hirudin in *E. coli* than the other three signal peptides, and the majority of the protein was transported to the medium.

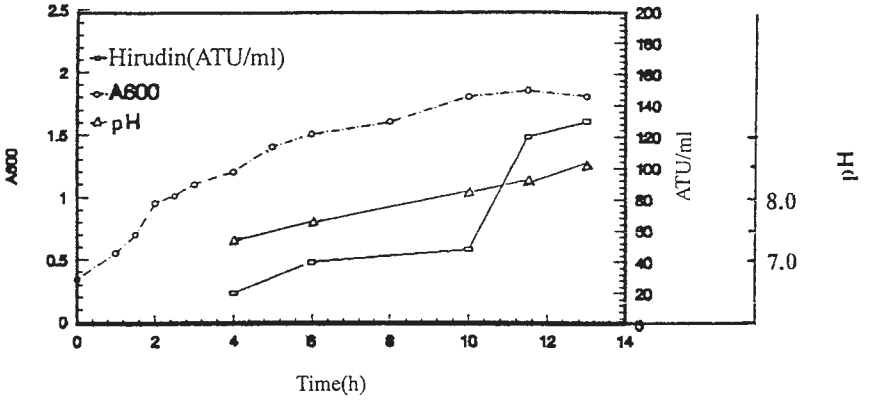

Fig. 2. Expression and secretion of hirudin. The cell culture was induced with 0.1 mmol/L of IPTG at 37°C when A_{600} is 0.6–0.8. The activity of hirudin was almost maximal at 10 h after induction. pH of the medium increased during this process.

Table 2
Antithrombin Activity of Four Mutants Containing Different Signal Peptides

	Activity (ATU/[mL·culture])[a]		
Signal peptide	Medium	Periplasm	Cytoplasm
bla	9.7	0.638	0.267
lpp	Negligible	Negligible	0.232
phoA	362.0	185.0	144.5
proteinA	7.4	11.83	0.619

[a]ATU, antithrombin activity unit.

Purification of HV2-K47

The expressed protein was purified (Fig. 3). High homogeneity of purified HV2-K47 was seen (Fig. 4). A single band corresponding to the dimer of HV2-K47 on 15% Tricine-polyacrylamide gel was evidenced. The recovery and purity of each step in purification are shown in Table 3. Because of its tolerance to low pH and high temperature, the product also could be heated to 70°C at pH 4.0 at the first step in order to remove a majority of the miscellaneous proteins and facilitate further purification.

Characterization of HV2-K47

The molecular weight of HV2-K47 determined by mass spectrometric analysis (Fig. 5) (MALDI-TOF MS; Hewlett-Packard) was 6909, which was very close to the molecular weight of 6907 calculated according to its amino acid sequence. This shows that the signal peptide was processed correctly.

The sequence of N-terminal 10 amino acids (ITYTDCTESG) analysis tallied completely with that designed (Applied Biosystems 491 Protein Sepuencer). The data for amino acid composition analysis were as follows:

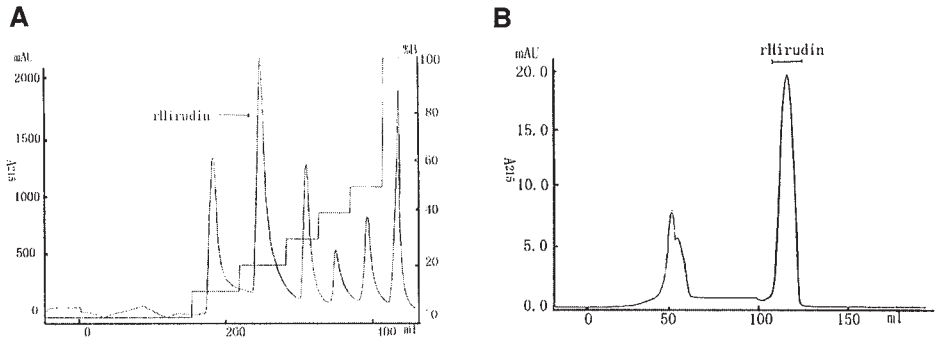

Fig. 3. **(A)** Purification of HV2-K47 DEAE Sepharose Fast Flow ion-exchange chromotography. Protein was eluted by graduations of NaCl from 0 to 0.5 mol/L; **(B)** Superdex 75 gel filtration chromatography. Fractions with activities were eluted by 2 to 3 vol of 20 mmol/L Tris-Cl (pH 7.5).

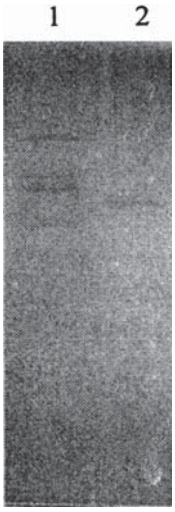

Fig. 4. Polyacrylamide gel electrophoresis (PAGE) analysis of the HV2-K47 (lane 1) low molecular weight marker (from top: 31.0, 20.4, 16.9, 14.4 kDa); lane 2, purified HV2-K47. Fifteen percent Tricine-PAGE was used. It showed a single band corresponding to the dimmer of HV2-K47.

Table 3
Purification Protocol of HV2-K47

Step	Activity recovery (%)	Fold purification
Pretreatment	91.96	1.06
DEAE-Sepharose FF	82.78	3.79
Superdex 75	56.16	38.32
C2/C18	25.10	50.19

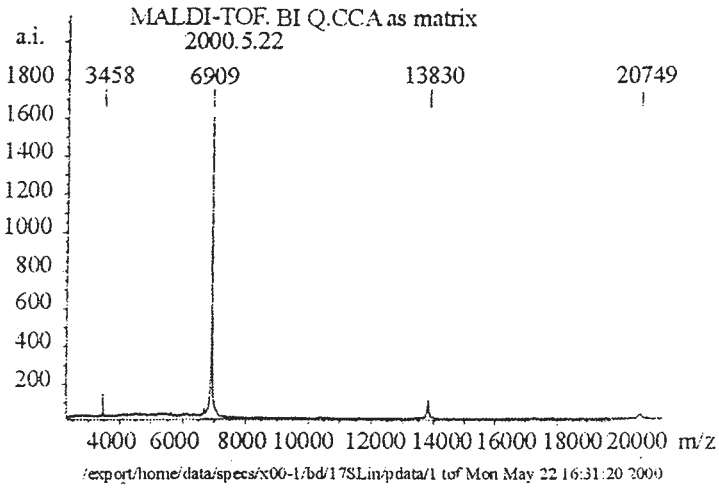

Fig. 5. Mass spectrometric analysis of purified HV2-K47. The molecular weight of HV2 is 6909, which is similar to the calculated molecular weight of 6907.

Asp 9 (8.66), Thr 5 (4.23), Ser 4 (3.68), Glu 11 (11.4), Pro 3 (3.22), Gly 10 (10.02), Val 2 (2), Leu 4 (3.18), Tyr 2 (1.31), phe 1 (0.912), Lys 4 (3.61), His 1 (1.14), Ile 3 (3.6). The data agreed with the expected value.

Our studies on the Lys-47 mutant further show that the Lys-47 may indeed be the key residue in hirudin. Our results demonstrate that the specific activity of HV2-K47 is 13,500 ATU/mg, which is two times higher than that of recombinant HV2 (6600 ATU/mg) and the same as that of native HV2 (13,000–14,000 ATU/mg) *(8)*. In addition, the HV2-K47 has extinction coefficients at 205 nm for 1 mg/mL solution of 30.3.

Hirudin is stable at low pH (p*I* = 3.9) and high temperature and is resistant to proteolysis *(9)*. Our studies demonstrated that it continues to have major activity at 70°C for 30 min at pH 4.0. Its stability further indicates that hirudin can be a valuable drug in the clinic.

As already described, at first we used the expression vector pET21a with the *lacI* repressor gene to express rHV2-K47, which must be induced by IPTG. However, to facilitate the fermentation, pET21a was replaced by vector pBV220 with cIts857 repressor gene, which should be induced by heat shock. Many factors are involved in protein expression, such as vector, promoter, and SD sequence. Our results show that signal peptide sequence has a distinct influence on the expression level of foreign proteins. According to the measurement of thrombin inhibitory activity, the *phoA* signal peptide is the best for secretion of hirudin in *E. coli* among the four tested signal peptides. This may be because *phoA* is more suitable to express small proteins, or because it is only appropriate in this expression system. Further research should be conducted to determine the optimal expression conditions.

Conclusion

We can conclude from the presented results that the recombinant HV2-K47 has similar activity to native HV2 and that signal peptide is an important element in the expression of HV2-K47. Furthermore, hirudin is applicable as a model to study the regulation of protein expression in genetic engineering because of its extreme stability. Although further studies are still needed, as the most potent inhibitor of thrombin, hirudin is a promising antithrombin drug.

Acknowledgments

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