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Expression and Purification of Hi-lys Peptide, a Recombinant Relevant to Hirudin

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Abstract A new fusion expression vector, pED-P8-*Hi-lys* was designed and constructed. It includes four parts, a 20 peptide sequence of hirudin that can maintain anticoagulant activity, the C-terminus of asparaginase as a fusion partner, basic octopeptide (KRKRKKSR) that makes the fusion partner easy to remove, and the unique acid-labile aspartyl-prolyl bond. It was transformed into *E. coli* BL-21 and the fusion protein (AnsB-C-P8-Hi-lys) was expressed effectively as inclusion bodies after inducing by lactose. The objective peptide Hi-lys was purified by means of cell disruption, washing, ethanol precipitation, acid hydrolysis, and DEAE-cellulose 52 column chromatography. The antithrombin activity of the purified Hi-lys peptide was about 50 ATU/mg by thrombin activity assays.

Key words hirudin, antithrombin, fusion expression, octopeptide(KRKRKKSR)

重组水蛭素相关肽 Hi-lys 的表达与纯化

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摘要 为开发一种新的有临床应用价值的抗血栓药物,根据水蛭素保持抗凝活性的 20 肽片段,设计并构建了水蛭素相关肽(Hi-lys)与天冬酰胺酶 C 端的融合表达系统.为方便目的肽与融合伙伴的分离,增加了富含带电序列的 8 肽(KRKRKKSR)及酸敏感的天冬氨酰-脯氨酸(Asp-Pro)位点,获得了表达质粒 pED-P8-*Hi-lys*.将其转化 *E. coli* BL-21,玉米浆培养基(kan^r)培养,乳糖诱导获得融合蛋白(AnsB-C-P8-Hi-lys)的高效表达.通过细菌裂解、包涵体洗涤、尿素溶解、乙醇沉淀、酸水解和 DEAE-纤维素 52 柱层析纯化获得目的肽 Hi-lys,用凝血酶测定法测得其抗凝活性为 50 ATU/mg.

关键词 水蛭素,抗凝活性,融合表达,八肽(KRKRKKSR)
中图分类号 Q78,R972

Thromboembolic diseases are one of major causes of morbidity and mortality in world, which has stimulated enormous researchers in pharmaceutical industry area to introduce new antithrombotic therapies. Hirudin, from blood-sucking leeches (Hirudo medicinalis), is a naturally direct thrombin inhibitor[1]. It can inhibit effectively the formations of thrombus in artery or vein and disseminated intravascular coagulation (DIC). Researches have proved that its anticoagulant activity is better than heparin and it has a highly application value to prevention and therapy of thrombus embolism disease[2]. As the limited resources of natural hirudin, researchers have studied to produce recombinant hirudins (r-hirudins) through gene technology and succeeded in expression using *E. coli* or yeast[3,4]. Recombinant hirudins, such as desirudin (Ciba Novartis), have been shown to be effective in treatment of heparin-induced thrombocytopenia (HIT) and in the prevention of thrombotic complications after hip or knee surgery[5].

When administered to human intravenously, half-life

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of hirudin is very short, 1—2 hours. It can be modified with bio-macromolecule to prolong its half-life[6]. PEG-Hirudin, a chemically defined conjugate of recombinant hirudin, is a highly selective direct thrombin inhibitor with a significantly longer duration of action permitting once daily subcutaneous administration[4,7]. Studies suggested that only a 20-amino acid peptide (hirulog, bivalirudin) that combined a fragment of the C-terminus with N-terminus fragment of hirudin had a high anticoagulant activity[8]. It has a variety of potential application as an alternative to heparin in the management of cardiovascular disease and related medical procedures, such as unstable angina (UA), myocardial infarction (MI), percutaneous transluminal coronary angioplasty (PTCA), and ischemic heart disease (IHD)[9,10].

Here, we describe a recombinant fusion protein approach for the production of a new antithrombus peptide relevant to hirudin, that was Pro-Lys-Met-$(Gly)_3$-Phe-Pro-Arg-Pro-$(Gly)_4$-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu, named Hi-lys. It included the 20 peptide sequence of hirudin and a short sequence to combine activated PEG. An asparaginase C-terminal fragment from *E. coli* was tailored to serve as a carrier moiety for Hi-lys peptide. The truncated asparaginase fragment in which unique acid-labile aspartyl-prolyl (Asp-Pro) bond was mutated into Asp-Ala*, termed AnsB-C (unpublished), is easily expressed to high levels in *E. coli* and accumulates in inclusion bodies[11]. A linker containing basic octopeptide (KRKRKKSR) and an extra Asp-Pro bond was inserted between the AnsB-C moiety and Hi-lys peptide.

1 Materials and Methods

1.1 Materials

Heat stable *Pfu* DNA polymerase was purchased from Promega. Restriction endonucleases were from Takara Co. DE-52 was from Waterman (USA). Acrylamide and Bis-acrylamide were from Bio Basic Inc. (Canada). Sodium dodecyl sulfate (SDS) and urea was purchased from AMRESCO. Coomassie blue G-250 was from Sigma. The recombination hirudin 65 peptides was expressed and purified in our previous study[12]. Most other chemicals, unless specified, were obtained from Sangon Co. (Shanghai, China). All the chemical reagents used in the experiment were analytical grade.

1.2 General operations of gene clone

General operations such as extraction of plasmid, cutting with restriction enzymes, ligation, conversion and other standard DNA method were performed according to Sambrook et al[13].

1.3 Construction of the AnsB-C-P8-Hi-lys fusion protein

A DNA fragment coding AnsB-C and Asp-Pro linker was obtained by PCR amplification with 5′ primer (5′-CCC CAT GGA TAC GCC ATT CGA TGT CTA-3′) located at 663 to 683 of asparaginase, and 3′ primer downstream of the termination signal (5′-GCC AAT CGG ATC CGC GTA CTG GTT GAA GAT CTT GG-3′) from chromosome DNA template of *E. coli*. This and all subsequent PCR reactions were carried out in 100 μl volumes using 100 ng of DNA template, primers at a concentration of 1 μmol/L in the standard reaction buffer (dNTP's 0.2 mmol/L; 50 mmol/L KCl; 10 mmol/L Tris-HCl, pH 9.0; 0.1% Triton X-100). Typically, the reaction was taken through 30 cycles with 30 s at 94 ℃, 2 min at 55 ℃ and 2 min at 72 ℃. The resulting DNA fragment was digested with restriction endonucleases *Nco* Ⅰ and *Hin*d Ⅲ, and subsequently inserted downstream of the T7Φ10 promotor of the expression vector pET28a (Novagen, Germany). The resulting plasmid was termed pED. Next, the recombinant plasmid pED-P8 was constructed by extending the C-terminal part of AnsB-C with a linker sequence containing the octopeptide in series, which was carried out by add-PCR. Briefly, a DNA fragment coding AnsB-C and the linker sequence was generated by add-PCR amplification with 5′ primer (5′-TAA TAC GAC TCA CTA TAG G-3′) located at 663 to 683 of asparaginase, and 3′ adding primer (5′-CC AAC AGA TCT TCA ACC AGT ACG AAA CGT AAA CGT AAA AAA TCT CGT GCG GAT CCG-3′) from the plasmid DNA template of pED. The resulting PCR fragment with *Nco* Ⅰ site located at the 5′ end to the sequence coding AnsB-C and *Bam*H Ⅰ site located just on an extra Asp-Pro bond sequence, was digested with *Nco* Ⅰ and *Bam*H Ⅰ, and inserted into the expression vector pED linearized with *Nco* Ⅰ and *Bam*H Ⅰ. The resulting expression vector was termed as pED-P8.

Hi-lys peptide gene was generated by PCR amplification using 5′ primer (5′-TAAAATG GAT CCG AAA ATG GGT GGT GGT TTT CCG CGC CCA GGT GGT-3′), 3′ primer (5′-CCCA AGC TTA CAG GTA TTC TTC CGG GAT TTC TTC GAA GTC ACC GTC-3′) and template oligonucleotide (5′-GT TTT CCG CGC CCA GGT GGT GGT GGT GAC GGT GAC TTC GAA GAA AT-3′). The resulting PCR fragment with *Bam*H Ⅰ site located at the 5′ end penultimate to the coding sequence of Hi-lys and *Hin*d Ⅲ site located just after the 3′ end to the termination codon TAA, was digested with *Bam*H Ⅰ and *Hin*d Ⅲ, and inserted into pED-P8 linearized with *Bam*H Ⅰ and *Hin*d Ⅲ. The resulting expression vector pED-P8-*Hi-lys* was used to transform *E. coli* BL21.

1.4 Expression and purification of AnsB-C-P8-Hi-lys fusion protein

Firstly, a single recombinant colony of *E. coli* BL21 containing pED-P8-*Hi-lys* was inoculated into selective LB liquid medium (50 μg/ml kanamycin) at 37 ℃ for 10 hours. Then this culture (as a seed culture) was transferred into corn liquid medium (inoculated 2% V/V). The bacteria were grown at 37 ℃ and the expression of the fusion protein AnsB-C-P8-Hi-lys was induced at an

A_{600nm} of 1.5 to 1.8 (for about four hours) by addition of lactose to a final concentration of 5 mmol/L. Continue culturing at 37 ℃ with shaking at 270 r/min for 8 hours, reached a final A_{600nm} of 2.6 to 3.2, and sampling for polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) to determine the expression level of the recombinant proteins.

The following steps were carried out at 4 ℃ unless otherwise specified. Cultures were centrifuged at 5 000 r/min for 15 min in a Bechman centrifuge and the supernatant discarded. The cell pellet was washed with Tris-HCl buffer containing 50 mmol/L Tris-HCl, pH 8.0, 2 mmol/L EDTA, lysed in buffer A (0.2‰ lysozyme, 0.5 % Triton X-100, 50 mmol/L Tris-HCl, pH 8.0, and 2 mmol/L EDTA) and stirred at 37 ℃ for 4 hours. Then, the incubation mixtures were centrifuged at 8 000 r/min for 20 min and the pellet washed with buffer B (0.2 % Triton-100, 50 mmol/L Tris-HCl, pH 8.0) and 2.0 mol/L urea respectively. The inclusion bodies were collected in the pellet by centrifugation as above and suspended in buffer C (4 mol/L urea, 100 mmol/L Tris-HCl, pH 8.0) overnight, centrifuged as above. The supernatant was added with equal volume of anhydrous ethanol, incubated at - 20 ℃ for one hour and then centrifuged at 8 000 r/min for 20 min. The supernatant was added continuously with three times volume ethanol and kept at - 20 ℃ overnight. The fusion protein was collected by centrifugation and taken for SDS-PAGE analysis.

1.5 Purification of objective peptide Hi-lys

In order to cleave the unique acid-labile asp-pro bond in the fusion protein, the purified AnsB-C-P8-Hi-lys was dissolved in 60 mmol/L hydrochloric acid to 6 % (W/V), and the solution was incubated at 50 ℃ for 60 hours. The cleavage reaction was terminated by the addition of 60 mmol/L sodium acetate, the solution pH was adjusted to 9.0 ~ 9.3 with 2mol/L sodium hydroxid, and then centrifuged at 12 000 r/min for 10 min. The supernatant was collected and applied to DE-52 column (26 mm × 300 mm) that was pre-equilibrated with 20 mmol/L phosphate buffer (equilibration buffer), pH 6.8, at a flow rate of 1.5 ml/min. The column was washed with 5 column volumes of the equilibration buffer and then eluted with a linear gradient buffer between 20 mmol/L and 500 mmol/L sodium chloride in equilibration buffer (240 ml × 240 ml). The effluent was monitored continuously at 280 nm and the peak fractions were taken for SDS-PAGE and antithrombin activity analysis. The sample containing Hi-lys was pooled and lyophilized.

1.6 SDS-PAGE analysis of the fusion protein and peptide

40 μl each fraction were added in 10 μl 5 × SDS-loading buffer containing 250 mmol/L Tris-HCl, pH 6.8, 500 mmol/L DTT, 10 % SDS, 0.5 % bromophenol blue, and 50 % glycerol, and heated at 100 ℃ for 5 min. Proteins were separated by 15 % polyacrylamide gel and detected by staining with Coomassie blue G-250. Peptides were separated by 20 % polyacrylamide gel according to the method of Laemmli[14].

1.7 Antithrombin activity assays of Hi-lys

Thrombin assays described by Markwardt[15] was used for determining the antithrombin activity. Since thrombin activity is standardized in National Institute of Health units, the antithrombin activity of Hi-lys can be expressed in antithrombin units (ATU). Aliquots of 0.005 —0.01 ml of Hi-lys (50 mg/ml in 0.05 mol/L Tris-HCl, pH 7.4) were added to 0.2 ml of a 0.5 % fibrinogen solution in Tris-HCl buffer. After mixing, a standardized thrombin solution (100 NIH units/ml) was gradually added in 0.005-ml aliquots (corresponding to 0.5 NIH units) in minute intervals at room temperature. The titration end point was reached when the fibrinogen coagulated within one minute.

2 Results

2.1 Construction and expression of the AnsB-C-P8-Hi-lys fusion protein

Using primers designed up- and down- stream of the ansB gene's C-terminal sequence (ansB-C) reported, the putative ansB-C gene with Asp-Pro sequence was amplified by PCR from isolated DNA of *E. coli* HB101. The expression vector was constructed by cloning the PCR fragment into the T7Φ10 promoter-based pET28a expression vector and designated pED. The ansB-C with an extra C-terminal KRKRKKSR and Asp-Pro sequence was amplified by adding-PCR from isolated pED plasmid DNA. The expression vector pED-P8 was constructed by cloning the PCR fragment into *Nco* Ⅰ and *Bam*H Ⅰ sites of pED. The final expression vector was constructed by cloning a 98 bp PCR fragment carrying the Hi-lys gene into pED-P8 and designated pED-P8-*Hi-lys* (Fig. 1). Isoelectric point (pI) and molecular weight (M_r) of the AnsB-C-P8-Hi-lys fusion protein, the AnsB-C-P8 fusion partner and Hi-lys were predicted through ProtParam software in www.expasy.com. Their pI were 5.48, 9.3 and 3.77 respectively; and M_r were 17.7 kD, 15.1 kD and 2.61 kD respectively. The amount of the AnsB-C-P8-*Hi-lys* fusion protein in engineering strains carrying pED-P8-*Hi-lys* increased throughout the entire growth phase of 10 hours even with lactose induction, whereas the highest level of the fusion protein was arrived 8 hours fermentation as judged by SDS-PAGE (Fig. 2).

2.2 Preparation and purification of the AnsB-C-P8-Hi-lys fusion protein

As the result showed in Fig. 2, when the recombinant plasmid pED-P8-Hi-lys was used to transform *E. coli* BL21, high-level expression of fusion protein, which was 40 % ~ 60 % in total proteins of bacteria (analyzed by Labworks 4 software), could be obtained after induction by lactose, as inclusion bodies. The fusion protein seemed easy to partially purify by cell disruption,

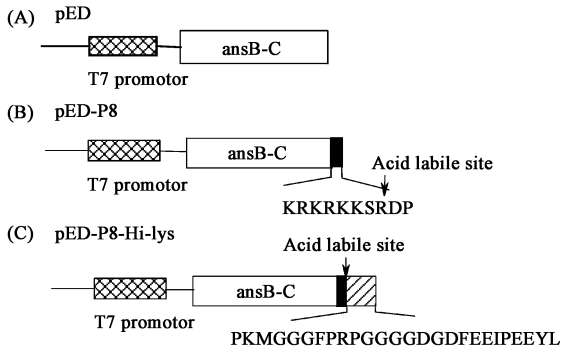

Fig. 1 Diagrams of the expression vectors pED for AnsB-C, pED-P8 for AnsB-C with an extra C-terminal KRKRKKSR sequence and acid labile Asp-Pro sequence, and pED-P8-*Hi-lys* for AnsB-C-P8-Hi-lys fusion protein
T7 promoter was represented in hatched boxes, and recombinant ansB-C gene were represented by open boxes, the KRKRKKSR sequence and Asp-Pro sequence was drawn as black and amino acids are represented by one-letter code

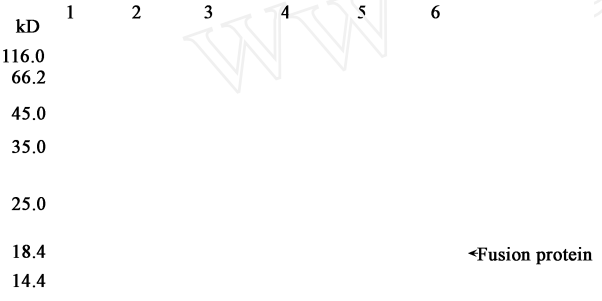

Fig. 2 SDS-PAGE analysis of the expression products in *E. coli*
1: Standard molecular weight marker;
2—6: Total proteins from *E. coli* with pED-P8-Hi-lys in 2, 4, 6, 8 and 10 hours respectively after lactose induction

washing and ethanol precipitation. The fusion protein with insertion of the KRKRKKSR sequence has a relatively high density and stability. Here the inclusion body materials can be washed with washing media containing 0.2% Triton X-100 and 2.0 mol/L urea. The inclusion body was purified to near homogeneity only by washing

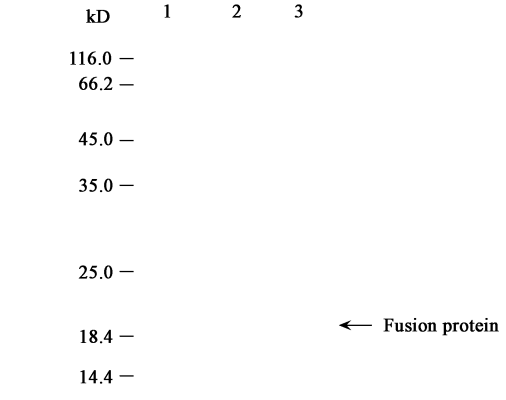

Fig. 3 SDS-PAGE analysis of purified AnsB-C-P8-Hi-lys
1: Standard molecular weight marker;
2: Total proteins from *E. coli* BL21 with pED-P8-Hi-ly after induction;
3: Purified AnsB-C-P8-Hi-lys fusion protein

pellets after disrupting bacteria, and alcohol precipitation (Fig. 3, lane 3), with the yield 2.6%～3.4% of the total proteins of bacteria (wet weight).

2.3 Preparation and purification of Hi-lys

As the result showed in Fig. 4, the fusion partner and Hi-lys were produced increasingly as hydrolysis time elongated. The hydrolysis was completed at about 72 hours. As the non-specific hydrolysis existed, the fusion protein could be cleaved into other parts. But digested at Asp-Pro bond was dominant. When the solution pH after hydrolysis was adjusted to 9.0～9.3, the solution began turbid and the precipitation was removed by centrifuging. The supernatant was separated by means of DE-52 chromatography (the results not be showed) and the objective peptide Hi-lys was purified (Fig. 5, lane 4) with the yield 1.7%～2.0% of the total protein weight of the inclusion body.

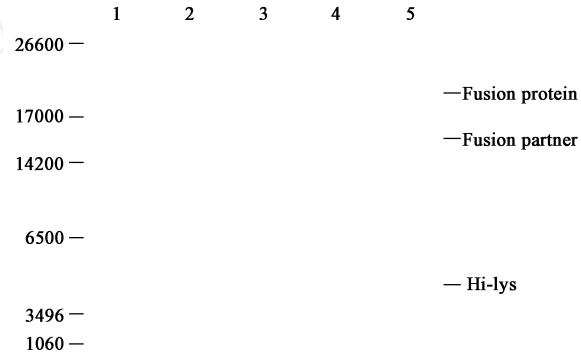

Fig. 4 SDS-PAGE analysis of hydrolysis of AnsB-C-P8-Hi-lys
1: Low molecular weight marker; 2: AnsB-C-P8-Hi-lys fusion protein;
3: Fusion protein hydrolyzed by hydrochloric acid for 23 huors;
4: Fusion protein hydrolyzed by hydrochloric acid for 56 hours;
5: Fusion protein hydrolyzed by hydrochloric acid for 72.5 hours

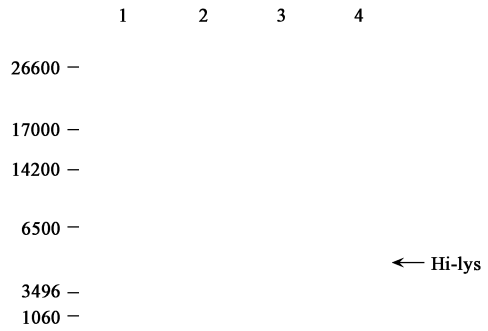

Fig. 5 SDS-PAGE analysis of purified Hi-lys peptide
1: Low molecular weight marker; 2: AnsB-C-P8-Hi-lys fnsion protein;
3: fusion protein hydroloyzed by hoydrochloric acid for 23hours;
4: Purified Hi-lys peptide

2.4 Antithrombin activity assays of Hi-lys

The result of thrombin assays showed that the purified Hi-lys peptide has antithrombin activity, about 200～300 ATU/ml (50 ATU/mg), while the 65 peptides hirudin as a control has the antithrombin activity of 7 500

ATU/mg.

3 Discussion

E. coli was an important host organism and widely used in industrial production because of easy cultivation, low cost, and high production potential. In our previous study, the recombination hirudin 65 peptides was efficiently expressed in *E. coli* using the *L*-asparaginase Ⅱ signal sequence and the product was secreted into the culture medium[12].

Considering of the short half-life of hirudin, we designed a novel polypeptide that retained the potent antithrombin activity of hirudin and could be modified with actived PEG. Expression of small polypeptide has often been unsuccessful because of their sensitivity to endogenous proteases in the engineered strain. In order to improve the expression rate we constructed a high effective expression vector pED to express the polypeptide, such as glucagon and glucagon like peptide-2, in form of fusion protein by using an asparaginase C-terminal fragments from *E. coli* as a carrier moiety, developed the method and technique to remove fusion partner[17] and studied the effect of culture conditions on plasmid stability of expression vector pED and analysed possible mechanisms[11]. As the result showed in Fig. 2, when the recombinant plasmid pED-P8-Hi-lys was used to transform *E. coli* BL21, high-level expression of fusion protein could be obtained as inclusion bodies, 40 %—60 % in total proteins of bacteria。

Several splice variants of VEGF(Vascular endothelial growth factor) contain basic octopeptide (KRKRKKSR) identified as a cell surface retention sequence and can be purified by haparin-Sepharose column chromatography[18,19]. So we supposed that the strongly basic octopeptide could be used as a purification tag for preparation of recombinant peptides. We added the basic octopeptide into the fusion partner, that make the isoelectric point of fusion partner (AnsB-C-P8) and Hi-lys be different significantly (9.3 and 3.77 respectively) while they were approach initially without the octopeptide, and then the purification process of Hi-lys be simplified. Furthermore the unique acid-labile Asp-Pro bond was added in the fusion protein that could be cleaved by acid to make Hi-lys release. Results tested our design: after cleavage with hydrochloric acid, Hi-lys peptide was released from the fusion protein, and the AnsB-C fusion partner with the octopeptide extension was subsequently removed by isoelectric point precipitation and DE-52 column chromatography.

The yield of Hi-lys was 1.7～2.0 % of the total protein weight of the inclusion body, showing that the purification procedure be successful. This suggests that this expression system might be useful for preparation of other biologically active peptides. Also, the result of thrombin assays supported that the purified Hi-lys peptide has antithrombin activity. It will allow us to determine the effectiveness of Hi-lys as a new antithrombotic agent in future experiments.

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