J Ind Microbiol Biotechnol (2012) 39:1487–1494
DOI 10.1007/s10295-012-1156-3

BIOTECHNOLOGY METHODS

Robust preparative-scale extracellular production of hirudin in *Escherichia coli* and its purification and characterization

Cuicui Huang · Xuerui Zhang · Jia Qu · Ping Zhang · Shuhua Tan

Received: 21 December 2011 / Accepted: 31 May 2012 / Published online: 31 July 2012

Abstract Hirudin variant III (HV3) is potentially useful in the prevention and treatment of cataracts. To prepare sufficient amounts of rHV3 for further preclinical studies, we developed an effective process for robust preparative-scale extracellular production of rHV3 in *Escherichia coli*. In a 7-l bioreactor, under the optimal fed-batch fermentation conditions, rHV3 was excreted into the culture supernatant and yielded up to 915 mg l^{-1}. Then, a four-step purification procedure was applied to the product, which included ultrafiltration, hydrophobic chromatography, anion-exchange chromatography, and preparative reversed-phase fast protein liquid chromatography (FPLC). The overall maximum recovery attained was 56 %, the purity reached at least 99 % as evaluated by HPLC analysis, the molecular weight was determined to be 7,011.10 Da by matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF/MS) analysis, and the pI was 4.46 as analyzed by isoelectric focusing. The N- and C-terminal sequence analysis confirmed the product homogeneity. The final product contained at most 10 pg of residual DNA per dose (0.2 mg) of rHV3 by high-sensitivity hybridization assay and at most 3 EU endotoxin protein/mg by limulus amebocyte lysate assay. Taken together, the rHV3 produced in multigram quantities in *E. coli* by this bioprocess meets the regulatory criteria for biopharmaceuticals and can be used as a drug candidate for preclinical studies.

Keywords Hirudin · Preparative-scale extracellular production · Fermentation

Abbreviations

ATU	Antithrombin units
FPLC	Fast protein liquid chromatography
HPLC	High-performance liquid chromatography
HV3	Hirudin variant III
MALDI-TOF	Matrix-assisted laser-desorption time-of-flight mass spectrometry

Introduction

Escherichia coli is usually the first choice host microorganism for the mass production of recombinant proteins of pharmaceutical and industrial interest. Despite the lack of post-translational modification and the existence of endotoxin, it has numerous desirable characteristics as a production host, such as fast cell growth, easy manipulation, straightforward high cell density fermentation, and capacity to hold over 50 % of foreign protein in total protein expression [41].

However, it has proven to be difficult to produce small eukaryotic polypeptides containing intramolecular disulfide bonds by employing cytoplasmic expression in *E. coli* because of their degradation by various proteases and difficulty in correctly forming the disulfide bridges in the cytoplasm [1, 21]. Compared to intracellular expression, direction of the peptide of interest to the periplasmic space or into the culture medium has multiple advantages, such as simplicity of purification, avoidance of protease attack, and absence of N-terminal methionine extension, as well as correct protein folding [3, 25]. Although there are

Cuicui Huang and Xuerui Zhang contributed equally to this work.

C. Huang · X. Zhang · J. Qu · P. Zhang · S. Tan (✉)
State Key Laboratory of Natural Medicines, China Pharmaceutical University, 24 Tongjia Street, 211# mailbox, Nanjing 210009, People's Republic of China
e-mail: tohike@hotmail.com

numerous examples showing secretion of various proteins, such as human leptin, staphylokinase(sak), hGCSF, hTPA, hEGF, hPTH, hGH, salmon calcitonin, and scFv antibody in *E. coli* [3, 23], very few of them have been commercialized because of the relatively low yield.

Hirudin is a typically secreted small polypeptide with three intramolecular disulfide bonds, which was initially isolated from *Hirudo medicinalis* [30]. Hirudin is a highly potent inhibitor of thrombin and two hirudin variants and a hirudin analogue have been approved for marketing as antithrombic drugs by the US Food and Drug Administration (FDA) [7, 36]. In addition to hirudin, two hirudin-like polypeptides HM1 and HM2 were identified from *Hirudinaria manillensis*, and the striking difference is the lack of a sulfated tyrosine residue in the C-terminal portion of the molecule which is replaced by aspartic acid [33, 37]. Interestingly, we have previously shown that recombinant hirudin variant III (rHV3) is potentially useful in the prophylaxis and treatment of cataract [26, 27]. Thus, to produce sufficient amounts of rHV3 for further preclinical studies, in this study, we developed an effective bioprocess for the robust preparative-scale extracellular production of active rHV3 with the expected characteristics in multigram quantities in *E. coli*.

Materials and methods

Bacterial strains and plasmids

Escherichia coli recombinant harboring plasmid pTASH [39] was used in this work for extracellular production of rHV3. The plasmid pTASH outlined in Fig. 1 contains the Tac promoter of pkk223-3 (Pharmacia Biotech), the high-copy replication origin of pUC18, as well as a modified L-ASP signal peptide gene sequence which was fused with the HV3 gene in the correct reading frame via a unique *Nhe*I restriction site at its 3′ end.

Fed-batch fermentation

The stock strain was inoculated into a 500-ml flask containing 200 ml of liquid LBA medium (LB medium supplemented with 100 μg ampicillin ml^{-1}) and cultured in a rotary shaker at 37 °C for 14 h at 220 rpm. Then, the culture was transferred to LBA medium at 2 % (v/v) for seed scale-up. After 8-h incubation at 37 °C, the seed culture was then transferred to 5 l of fermentation medium in a 7-l bioreactor (Laboratory Fermenter Type L1523, Bioengineering AG, Switzerland) at 4 % (v/v) for fermentation. The fermentation medium contained 10 g tryptone l^{-1}, 5 g yeast extract l^{-1}, 40 g monosodium glutamate l^{-1}, 10 g malt extract powder l^{-1}, 6.7 g KH_2PO_4 l^{-1}, 7.57 g $Na_2HPO_4{\cdot}12H_2O$ l^{-1}, and was supplemented with 100 mg ampicillin l^{-1}, pH 6.5. During fermentation, the pH was maintained at 6.3–7.5 by controlled addition of NH_4OH or HCl, the temperature was set at 37 °C, and the dissolved oxygen was maintained at 30–60 % air saturation level.

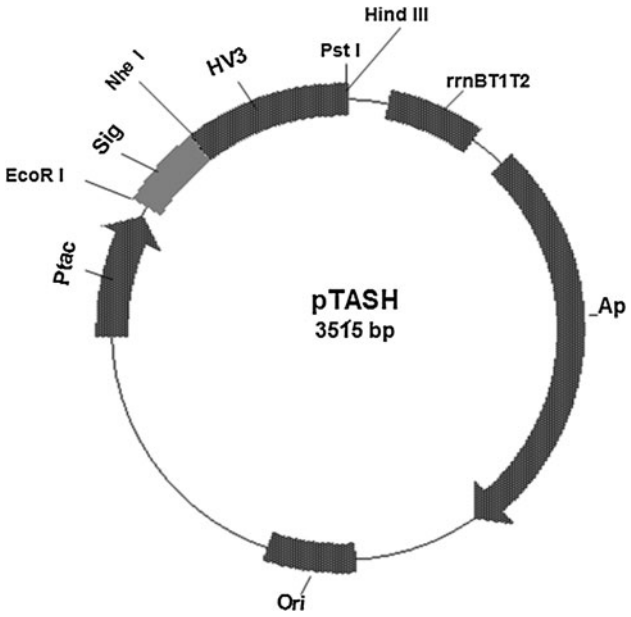

Fig. 1 Construction of secretory expression vector pTASH. *Ptac* Tac promotor, *Ap* ampicillin resistance gene, *HV3* hirudin variant 3 gene, *Sig* degenerate L-asparaginase II signal sequence, *Ori* replication origin of pUC plasmid, *rrnBT1T2* transcription terminator

After fermentation for ca. 3 h, the OD_{600nm} reached 3. Then, the fed-batch stage was started by supplementing with feed medium I (100 g malt extract powder l^{-1}) at a rate of ca. 150 ml h^{-1} for ca. 4 h, and the dissolved oxygen was controlled at 40–60 % of air. Thereafter, feed medium II (33.3 g tryptone l^{-1}, 16.7 g yeast extract l^{-1}, 133 g monosodium glutamate l^{-1}, 100 g malt extract powder l^{-1}) was supplemented at a rate of ca. 100 ml h^{-1} for ca. 2 h until the OD_{600nm} reached 9, then the fermentation was starved for ca. 2 h. Subsequently, it was supplemented with feed medium II at ca. 50 ml h^{-1} until the volumetric productivity reached a plateau. After completion of fermentation, the culture supernatant was collected by centrifugation at 12,000×*g* for 20 min at 4 °C, and frozen at −20 °C.

Protein purification

The culture supernatant was filtered through a 0.22-μm ultrafiltration membrane (Mini Pellicon GVPP, Milipore) and the filtrate was concentrated by using a 5-kDa MWCO ultrafiltration membrane (Mini Pellicon Biomax-5 C, Milipore). The concentrated filtrate was adjusted to pH 5.0–6.0

and loaded onto a Diaion HP20 (Mitsubishi Chemical, JP) column (ø5.5 × 60 cm) pre-equilibrated with 20 mM acetic acid. The column was washed thoroughly with 20 mM acetic acid, 50 mM Tris–HCl (pH 8.5), and 20 mM acetic acid containing 10 % (v/v) isopropanol sequentially. Then, elution was performed with 20 mM acetic acid containing 30 % (v/v) isopropanol, and active fractions were pooled for further purification.

The pooled fractions were slowly adjusted to pH 6.0 with 1 M piperazine and concentrated by using a 5-kDa MWCO ultrafiltration membrane (Labscale TFF Biomax 5 k A, Milipore), then loaded onto a DEAE-cellulose DE52 column (ø1.0 × 60 cm) pre-equilibrated with 20 mM piperazine (pH 6.0) and washed with the same buffer. Thereafter, elution was conducted with 20 mM piperazine (pH 6.0) in a linear gradient of 0–0.3 M NaCl.

Preparative reversed-phase separation was conducted using a Kromasil 5-C18 column (10 mm × 250 mm) on Bio-Rad BioLogic DuoFlow Pathfinder 20 FPLC system with a QuadTec UV/Vis detector (BioRad). Solvent A was 0.1 % trifluoroacetic acid in H_2O. Solvent B was 0.1 % trifluoroacetic acid in acetonitrile. The gradient went from 15 to 35 % B in 0–40 min and 30 to 100 % B in 40–45 min at a flow rate of 2 ml min^{-1}.

Antithrombin activity analysis

Antithrombin activity was measured quantitatively by means of titration of a solution of thrombin, and the thrombin neutralizing activity of the samples was expressed as antithrombin units (ATU). One ATU is the amount of test sample that neutralizes one NIH unit of thrombin. The test was performed as follows: 0.2 ml of a standard solution of 0.05 % bovine fibrinogen in Tris–HCl buffer pH 7.4 was incubated at 37 °C in the presence of 0.01–0.1 ml of the sample solution. Thereafter, aliquots of 5 µl (0.5 NIH units) of a 100 NIH units ml^{-1} thrombin standard solution were added progressively at an interval of 1 min and mixed gently. The end point of the titration was considered reached when a fibrin clot formed within 1 min.

Tricine/SDS-PAGE analysis

Tricine/SDS-PAGE analysis was performed according to Schagger [34]. Low molecular weight marker was obtained from Fermentas. Gels were stained with Coomassie Brilliant Blue R-250.

HPLC analysis and protein quantitation

The sample was applied to an Inertsil ODS-SP C18 column (4.6 × 250 mm) equilibrated with 0.1 % trifluoroacetic acid in H_2O on a Shimadzu LC-2010 HPLC system. Solvent A was 0.1 % trifluoroacetic acid in H_2O. Solvent B was 0.1 % trifluoroacetic acid in acetonitrile. The product was eluted with a linear gradient of acetonitrile from 15 to 40 % B in 0–40 min and from 40 to 100 % B in 40–45 min with a flow rate of 1 ml min^{-1}. Protein concentration of the expressed product was determined by comparing sample peak area to the reference standard [32].

Isoelectric focusing

Isoelectric focusing (IEF) was performed on a Bio-Rad Model 111 Mini IEF Cell, and pI was determined by comparison with the migration of standard pI marker (Broad pI Kit, pH 4.45–9.6) in a Bio-Lyte ampholytes PAGE plate, pH 3–10.

MALDI-TOF/MS analysis

Matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF/MS) analysis was performed on 4800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). The purified product was diluted with 0.1 % TFA at different levels, such as 1× and 10×. Then, a matrix comprising α-cyano-4-hydroxycinammic acid at 5 mg/ml in 50 % acetonitrile containing 0.1 % TFA was used. After 0.5 µl sample was spotted on the well of the MALDI target under a magnetic field, and 0.5 µl matrix solution was added dropwise to the sample. After air-drying, the molecular weight was analyzed using MALDI-TOF/MS. During MS analysis, the linear high mass mode was operated, and scanning range was from 3,000 to 20,000 Da. The laser power was set at 6,000. The mass spectra were acquired during 1,000 laser shots after being calibrated using an internal calibration generated from the ion signal of peptide mixtures of myoglobin (1,6951.4 Da, Sigma).

N- and C- terminal amino acid sequence analysis

The N-terminal amino acid sequence of rHV3 was determined by Edman degradation with a Procise® sequencing system, model 491(Applied Biosystems, Framingham, MA, USA). The C-terminal amino acid sequence analysis was performed on a Thermo LTQ Velos bioanalytical mass spectrometry system (Thermo, Finnigan, USA)

Detection of residual *E. coli* DNA

The chemiluminescent slot-blot hybridization method [10] was utilized for the quantitative determination of residual *E. coli* DNA in the purified rHV3 product by using DIG

High Prime DNA Labeling and Detection Starter Kit I as well as positively charged nylon membranes (Roche Applied Science, Indianapolis, IN, USA).

Endotoxin assay

Endotoxin level was tested by using a standard limulus amebocyte lysate (LAL) assay (Zhanjiang Bokang Marine Biological Co., Ltd., China) following the manufacturer's protocol.

Results

Fed-batch fermentation

Escherichia coli recombinant [39] harboring plasmid pTASH outlined in Fig. 1 was fed-batch cultivated under optimal conditions in a 7-l bioreactor for extracellular production of rHV3 resulting in the accumulation of extracellular antithrombin activity up to 1.2×10^4 ATU ml^{-1} within 24 h (Fig. 2). Since the finally purified product showed a specific activity of 13,118 ATU mg^{-1}(see Table 1), the yield was thus calculated to be 915 mg l^{-1}. From the process curves of fed-batch fermentation, it can be seen that the cell density was increased to a higher level by feeding at the first phase, and as the cell growth reached a plateau the product expression was initiated and kept increasing until the fermentation was completed. Nevertheless, to achieve high-level expression, efforts should be made to maintain a favorable dissolved oxygen level, nutrient balance, and relatively constant pH during the period of fermentation.

Product purification from culture medium

Cell-free culture supernatant was prepared by centrifugation and ultrafiltration. Thereafter, the product was highly purified from the clarified supernatant in four sequential steps, which included ultrafiltration, Diaion HP20 hydrophobic chromatography, DEAE-cellulose DE52 anion-exchange chromatography, and preparative RP-FPLC. In a typical case, 683 mg of final product with a purity at least 99 % was obtained from 1.3 l culture supernatant by using this purification approach. The maximum overall recovery rate was 56 %, the final product showed a specific activity of 13,118 ATU mg^{-1}, and the % purity of samples at every step of the purification process was independently determined by comparing the rHV3 peak area to the total protein peak areas by analytical HPLC (Table 1).

Tricine/SDS-PAGE analysis of the final product was conducted on 18 % (w/v) resolving gel and stained with Coomassie Brilliant Blue R-250 (Fig. 3). In addition, the purity of the final product was further determined to be at least 99% by HPLC analysis (Fig. 4).

Characterization of the final product

MALDI-TOF/MS analysis on the final purified product gave a mass value of 7,011.10 Da (Fig. 5), which was similar to the theoretically calculated value of 7,025.61 Da (DNAsis software Ver2.5, Hitachi Software Engineering Co., Ltd). Comparison with the mass value of ca. 14 kDa

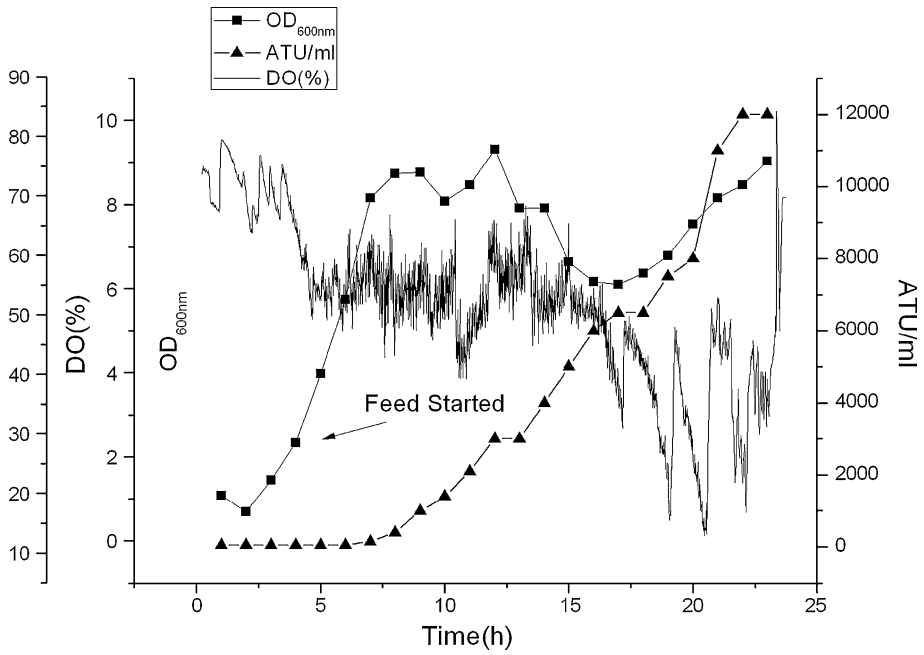

Fig. 2 Process curves of fed-batch fermentation under the optimal conditions in a 7-l stirred tank bioreactor. *ATU/ml* antithrombin activity in culture medium, OD_{600nm} optical density at 600 nm, *DO(%)* dissolved oxygen in air saturation

Table 1 Purification of rHV3 from culture medium

Purification step	Total protein (mg)	Total activity ($\times 10^5$ ATU)	Specific activity (ATU mg^{-1})	Purity (%)	Recovery (%)
Culture supernatant	5,452	160	3,082	10	100
Ultrafiltration	4,438	142	3,210	10	89
Diaion HP20	3,198	123	3,846	31	77
Ultrafiltration	2,102	110	5,233	47	69
DE-52 cellulose	1,024	102	8,499	85	64
C18 RP-FPLC	683	89.6	13,118	99	56

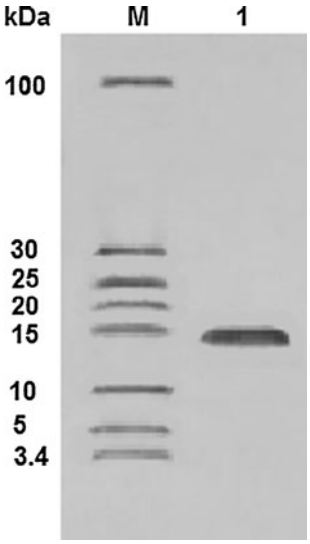

Fig. 3 Analysis of the purified rHV3 by tricine/SDS-PAGE on 18 % (w/v) resolving gel; the gel was stained with Coomassie Brilliant Blue R-250. *M* MW marker (kDa), *1* finally purified rHV3

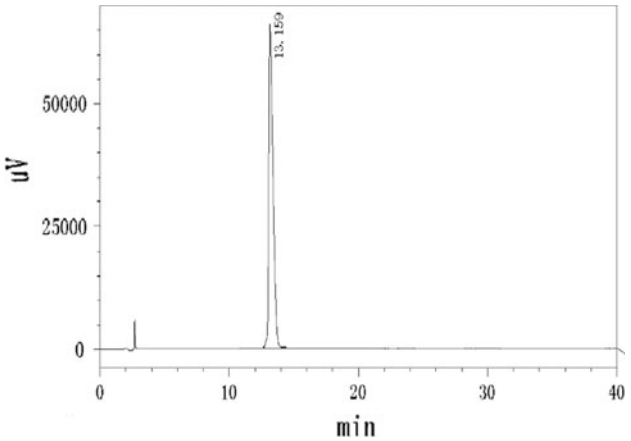

Fig. 4 HPLC analysis of the finally purified rHV3 product by using an Inertsil ODS-SP C18 column (4.6 × 250 mm) on a Shimadzu LC-2010 HPLC system. Solvent A was 0.1 % trifluoroacetic acid in H_2O. Solvent B was 0.1 % trifluoroacetic acid in acetonitrile. The product was eluted with a linear gradient of acetonitrile from 15 to 40 % B in 0–40 min and from 40 to 100 % B in 40–45 min with a flow rate of 1 ml min^{-1}

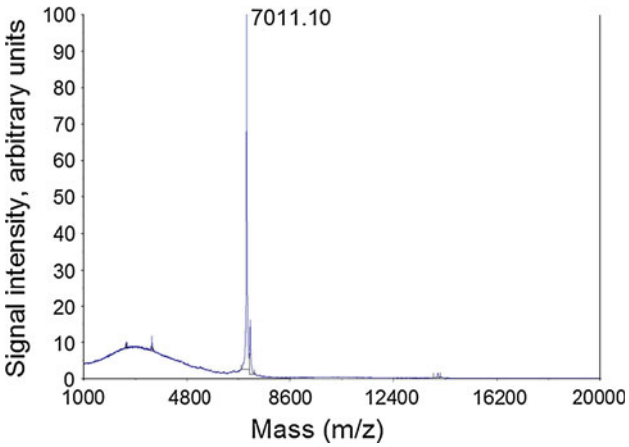

Fig. 5 MALDI-TOF/MS analysis on a 4800 Proteomics Analyzer (Applied Biosystems). The mass spectra were acquired during 1,000 laser shots after being calibrated using an internal calibration generated from the ion signal of peptide mixtures of myoglobin (16,951.4 Da, Sigma)

(Fig. 3) obtained by tricine/SDS-PAGE analysis indicated that the secreted product formed a dimer during the expression process. A similar phenomenon was also observed by another group [30], and it was speculated that the dimer formation could be caused by the strong hydrophobic interaction between two hirudin molecules.

The first 15 N-terminal amino acid residues of the product were determined to be N-ITYTDCTESGQNLCL-, and the C-terminal amino acid residues were -PQSHNQGDFEPI-PEDAYDE-COOH, which were completely consistent with those of natural HV3.

In addition, the isoelectric point of the product was determined to be 4.46 as compared to the standards that migrated in an adjacent lane on the Bio-Lyte ampholytes PAGE plate with a gradient range of pH 3–10 (Fig. 6), and it was very close to the theoretical value of 4.02 calculated by DNASTAR software (DNASTAR, Inc., Madison, WI).

Quantitation of residual *E. coli* DNA

By high-sensitivity hybridization assay, the finally purified product contained less than 10 pg of residual DNA per

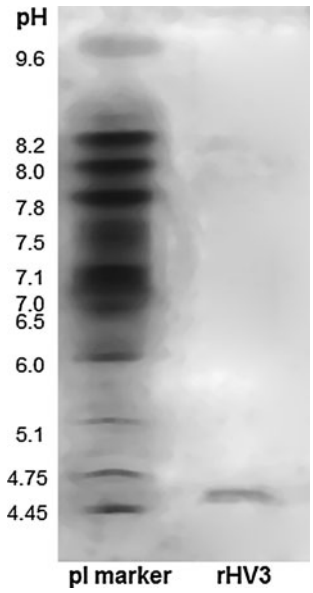

Fig. 6 pI measurement by using Bio-Lyte ampholytes PAGE plate with a gradient range of pH 3–10

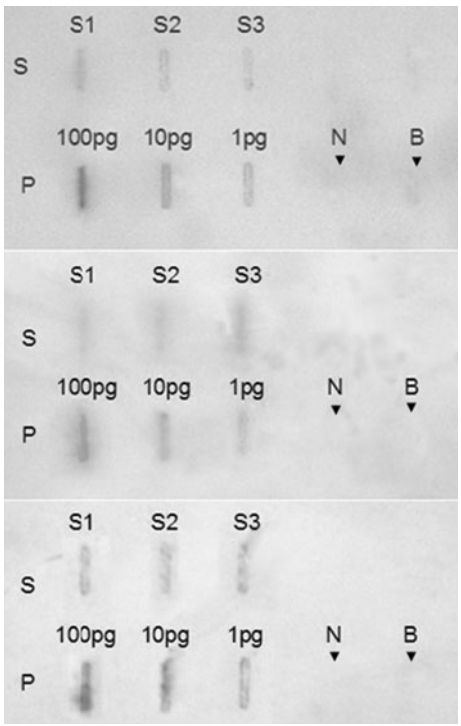

Fig. 7 Chemiluminescent hybridization assay for quantitative determination of residual *E. coli* DNA in purified rHV3 products. *P* DNA positive control, *S* test samples (0.2 mg rHV3 product), *N* negative control, *B* blank

0.2 mg rHV3 (Fig. 7), and it was below the safety limit 100 pg per dose suggested by the FDA for residual DNA in recombinant biopharmaceuticals [31], as the highest dose in our preclinical studies for prevention and treatment of cataract was set as 0.2 mg rHV3 per dose.

Endotoxin examination

The endotoxin detected in the final purified product by LAL assay was at most 3 EU per mg of protein.

Discussion

Thus far, various host organisms have been employed to express hirudin and its analogs either intracellularly or extracellularly, which include bacteria (*E. coli*, *Streptomyces lividans*, *Lactococcus lactis*) [2, 5, 8, 18], filamentous fungus (*Acremonium chrysogenum*) [29], transgenic plant [6, 28], transgenic mice milk [40], and yeasts such as *Saccharomyces cerevisiae* [4, 11, 13, 14, 16, 22], *Pichia pastoris* [32], and *Hansenula polymorpha* [12]. However, of these expression systems, only *E. coli* and *S. cerevisiae* organisms are accepted by regulatory authorities for recombinant production in the pharmaceutical industry, whereas the commonly used *P. pastoris* is usually preferred in fundamental research for the structural and functional analysis of proteins [35].

Hirudin and its analogs have been extracellularly expressed in *S. cerevisiae* at high level by fed-batch fermentation; however, the major drawback to this system is the long fermentation period, because it would inevitably increase the manufacturing cost and processing complexity. For example, in the case of rHV2-Lys 47, although it could be extracellularly expressed in *S. cerevisiae* at the high level of 500 mg l^{-1} by high cell density fed-batch fermentation, approximately 60 h was required for the overall fermentation period [22]. As another example, when hirudin was produced in GAL1-disrupted *S. cerevisiae* by optimal fed-batch culture, about 70 h was needed for the fermentation to achieve a yield of 62.1 mg l^{-1} [13].

In comparison to the *S. cerevisiae* system, the remarkable advantage in utilizing the *E. coli* system for the production of recombinant proteins is the short fermentation period, typically less that about 24 h. Thus, many efforts have been made to intracellularly express hirudin and its analogs in *E. coli* by employing various approaches. For example, hirudin variant 1 (f-HV1) which was fused to porcine adenylate kinase protein could be produced intracellularly at high level as an inclusion body in *E. coli* by temperature shift coupled with a high cell density fermentation technique [19, 20]; however, the product appeared to be inactive and no procedure for subsequent downstream cleavage and refolding was included. Another approach was to express hirudin-CBD-intein fusion protein in *E. coli*; however, the yield was quite low and not all

produced hirudin molecules possessed the correct pairing of cysteine residues [15]. Besides, a chimeric protein of human Annexin V fused to hirudin C-terminal segment free of intramolecular disulfide bond was designed and intracellularly expressed in *E. coli*, yet the expression was modest [42]. In addition, Liu et al. [17] developed a novel approach for direct expression of sulfohirudin in *E. coli* by genetically encoding the modified amino acid- sulfotyrosine in response to the amber nonsense codon TAG. In any event, these approaches appear to be unsuitable for scaling up to industrial production.

Secretory expression offers an attractive approach for the production of hirudin in *E. coli* owing to its multiple unique advantages. Initially, the phoA signal peptide was utilized to direct the secretion of hirudin in *E. coli*, two processed proteins were isolated from the periplasmic fraction, and one of them was identical to desulfatohirudin and also had similar biological properties [5]. Thereafter, Nippon Mining Company patented a secretory *E. coli* system for high-level extracellular production of various hirudin variants and a chimeric HV1/HV3 analog by using phoA signal peptide [24], and in the case of HV1C3, the yield of correctly processed product accumulated in the culture medium reached up to 507 mg l^{-1} after fermentation for 24 h. Besides, in our previous studies [9, 39], a novel secretory system for the extracellular secretion of HV3 was developed by using the L-asparaginase II signal sequence, and by fed-batch fermentation at bioreactor scale, bioactive rHV3 and its muteins could be efficiently secreted into the culture medium, and yielded up to 8.5×10^3 ATU ml^{-1} [38]. In this study, the yield was further improved and reached up to 1.2×10^4 ATU ml^{-1} after fermentation for only 24 h by the optimal fed-batch fermentation. Interestingly, no inducer such as IPTG or lactose was required for high-level secretory expression although the vector was constructed on the basis of Tac promoter. A possible reason is that the malt extract contained in the medium includes certain unknown components which may act as the inducer or the host cell is *lacI*$^-$. Subsequently, we developed an effective four-step purification procedure which consisted of ultrafiltration, hydrophobic chromatography, anion-exchange chromatography, and preparative reversed-phase FPLC. With this procedure, the product could be purified to homogeneity with an overall recovery of 56 %, and the product purity reached at least 99 % as evaluated by HPLC analysis. Biochemical analyses revealed that the product had the same characteristics as those of the wild-type HV3. In addition, the results also indicated that this purification procedure might enable the efficient removal of the residual *E. coli* DNA and endotoxin to allow the final product to be acceptable for pharmaceutical use.

In conclusion, the bioprocess described here offers an effective approach for the robust preparative-scale extracellular production of rHV3 in *E. coli* at low cost, and the product meets the regulatory requirement to be used as a drug candidate for preclinical studies.

Acknowledgments This work was supported by the Major Drug Innovation of National Eleventh Five-Year Major Project of China (2009ZX09103-653), National Natural Science Foundation of China (30873191). N- and C- terminal sequence analyses were conducted in the Research Centre for Proteome Analysis, Key Lab of Proteomics, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China.

References

1. Bardwell JC (1994) Building bridges: disulphide bond formation in the cell. Mol Microbiol 14:199–205
2. Bender E, Vogel R, Koller KP, Engels J (1990) Synthesis and secretion of hirudin by *Streptomyces lividans*. Appl Microbiol Biotechnol 34:203–207
3. Choi JH, Lee SY (2004) Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl Microbiol Biotechnol 64:625–635
4. Courtney M, Loison G, Lemoine Y, Riehl-Bellon N, Degryse E, Brown SW, Cazenave JP, Defreyn G, Delebassee D, Bernat A et al (1989) Production and evaluation of recombinant hirudin. Semin Thromb Hemost 15:288–292
5. Dodt J, Schmitz T, Schafer T, Bergmann C (1986) Expression, secretion and processing of hirudin in *E. coli* using the alkaline phosphatase signal sequence. FEBS Lett 202:373–377
6. Giddings G, Allison G, Brooks D, Carter A (2000) Transgenic plants as factories for biopharmaceuticals. Nat Biotechnol 18:1151–1155
7. Greinacher A, Warkentin TE (2008) The direct thrombin inhibitor hirudin. Thromb Haemost 99:819–829
8. Guo J, Wang H, Gu J (1998) Expression and purification of recombinant hirudin. Zhonghua Xue Ye Xue Za Zhi 19:146–148
9. Huang XD, Tong WW, Fan XL, Li ZH (2007) Application of HPLC-ESI-ITMS in the quality control of carboxyterminal sequence confirmation for the recombinant DNA product Hirudin Variant 3. J Pharm Biomed Anal 43:533–539
10. Ji X, Lee K, DiPaolo B (2002) High-sensitivity hybridization assay for quantitation of residual *E. coli* DNA. Biotechniques 32:1162–1167
11. Kim MD, Han KC, Kang HA, Rhee SK, Seo JH (2003) Coexpression of BiP increased antithrombotic hirudin production in recombinant *Saccharomyces cerevisiae*. J Biotechnol 101:81–87
12. Kim MD, Kang HA, Rhee SK, Seo JH (2001) Effects of methanol on expression of an anticoagulant hirudin in recombinant *Hansenula polymorpha*. J Ind Microbiol Biotechnol 27:58–61
13. Kim MD, Lee TH, Lim HK, Seo JH (2004) Production of antithrombotic hirudin in GAL1-disrupted *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 65:259–262
14. Kim MD, Rhee SK, Seo JH (2001) Enhanced production of anticoagulant hirudin in recombinant *Saccharomyces cerevisiae* by chromosomal delta-integration. J Biotechnol 85:41–48
15. Kochanowski R, Kotlowski R, Szweda P (2006) Novel method of expression and purification of hirudin based on pBAD TOPO, pTYB12 vectors and gene synthesis. Protein Expr Purif 50:25–30
16. Lehman ED, Joyce JG, Bailey FJ, Markus HZ, Schultz LD, Dunwiddie CT, Jacobson MA, Miller WJ (1993) Expression, purification and characterization of multigram amounts of a recombinant hybrid HV1-HV2 hirudin variant expressed in *Saccharomyces cerevisiae*. Protein Expr Purif 4:247–255

17. Liu CC, Schultz PG (2006) Recombinant expression of selectively sulfated proteins in *Escherichia coli*. Nat Biotechnol 24: 1436–1440
18. Lv J, Huang C, Zhang X, Tan S (2012) Extracellular secretion of anticoagulant peptide hirudin in *Lactococcus lactis* using SP310mut2 signal peptide. Biotechnol Lett 34:61–65
19. Matsui T, Sato H, Yamamuro H, Misawa S, Shinzato N, Matsuda H, Takahashi J, Sato S (2008) High cell density cultivation of recombinant *Escherichia coli* for hirudin variant 1 production. J Biotechnol 134:88–92
20. Matsui T, Sato H, Yamamuro H, Shinzato N, Matsuda H, Misawa S, Sato S (2008) High cell density cultivation of recombinant *E. coli* for hirudin variant 1 production by temperature shift controlled by pUC18-based replicative origin. Appl Microbiol Biotechnol 80:779–783
21. Maurizi MR (1992) Proteases and protein degradation in *Escherichia coli*. Experientia 48:178–201
22. Mendoza-Vega O, Hebert C, Brown SW (1994) Production of recombinant hirudin by high cell density fed-batch cultivations of a *Saccharomyces cerevisiae* strain: physiological considerations during the bioprocess design. J Biotechnol 32:249–259
23. Mergulhao FJ, Summers DK, Monteiro GA (2005) Recombinant protein secretion in *Escherichia coli*. Biotechnol Adv 23:177–202
24. Misawa S, Matsuda H, Inoue Y, Furuya H (1996) Production of a new hirudin analog and anticoagulant pharmaceutical composition containing the same. US Patent 5,516,656
25. Ni Y, Chen R (2009) Extracellular recombinant protein production from *Escherichia coli*. Biotechnol Lett 31:1661–1670
26. Ou Y, Geng P, Liao GY, Zhou Z, Wu WT (2009) Intracellular GSH and ROS levels may be related to galactose-mediated human lens epithelial cell apoptosis: role of recombinant hirudin variant III. Chem Biol Interact 179:103–109
27. Ou Y, Liao GY, Wu WT (2008) Potential use of hirudin in diabetic cataract: a study of galactose mediated human lens epithelial cells injury. Chem Biol Interact 173:141–147
28. Parmenter DL, Boothe JG, van Rooijen GJ, Yeung EC, Moloney MM (1995) Production of biologically active hirudin in plant seeds using oleosin partitioning. Plant Mol Biol 29:1167–1180
29. Radzio R, Kuck U (1997) Efficient synthesis of the blood-coagulation inhibitor hirudin in the filamentous fungus *Acremonium chrysogenum*. Appl Microbiol Biotechnol 48:58–65
30. Riehl-Bellon N, Carvallo D, Acker M, Van Dorsselaer A, Marquet M, Loison G, Lemoine Y, Brown SW, Courtney M, Roitsch C (1989) Purification and biochemical characterization of recombinant hirudin produced by *Saccharomyces cerevisiae*. Biochemistry 28:2941–2949
31. Riggin A, Luu VT, Lobdell JK, Wind MK (1997) A non-isotopic probe-hybridization assay for residual DNA in biopharmaceuticals. J Pharm Biomed Anal 16:561–572
32. Rosenfeld SA, Nadeau D, Tirado J, Hollis GF, Knabb RM, Jia S (1996) Production and purification of recombinant hirudin expressed in the methylotrophic yeast *Pichia pastoris*. Protein Expr Purif 8:476–482
33. Scacheri E, Nitti G, Valsasina B, Orsini G, Visco C, Ferrera M, Sawyer RT, Sarmientos P (1993) Novel hirudin variants from the leech *Hirudinaria manillensis*. Amino acid sequence, cDNA cloning and genomic organization. Eur J Biochem 214:295–304
34. Schagger H (2006) Tricine-SDS-PAGE. Nat Protoc 1:16–22
35. Schmidt FR (2004) Recombinant expression systems in the pharmaceutical industry. Appl Microbiol Biotechnol 65:363–372
36. Sohn JH, Kang HA, Rao KJ, Kim CH, Choi ES, Chung BH, Rhee SK (2001) Current status of the anticoagulant hirudin: its biotechnological production and clinical practice. Appl Microbiol Biotechnol 57:606–613
37. Sohn JH, Kang HA, Rao KJ, Kim CH, Choi ES, Chung BH, Rhee SK (2001) Current status of the anticoagulant hirudin: its biotechnological production and clinical practice. Appl Microbiol Biotechnol 57:606–613
38. Tan S, Wu W, Li X, Cui L, Li B, Ruan Q (2007) Enhanced secretion of adhesive recognition sequence containing hirudin III mutein in *E. coli*. Mol Biotechnol 36:1–8
39. Tan S, Wu W, Liu J, Kong Y, Pu Y, Yuan R (2002) Efficient expression and secretion of recombinant hirudin III in *E. coli* using the L-asparaginase II signal sequence. Protein Expr Purif 25:430–436
40. Yen CH, Yang CK, Chen IC, Lin YS, Lin CS, Chu S, Tu CF (2008) Expression of recombinant hirudin in transgenic mice milk driven by the goat beta-casein promoter. Biotechnol J 3:1067–1077
41. Yoon SH, Kim SK, Kim JF (2010) Secretory production of recombinant proteins in *Escherichia coli*. Recent Pat Biotechnol 4:23–29
42. Yuan H, Yang X, Hua ZC (2004) Optimization of expression of an annexin V-hirudin chimeric protein in *Escherichia coli*. Microbiol Res 159:147–156