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Enhanced Expression and Primary Purification of Soluble HBD3 Fusion Protein in *Escherichia coli*

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Abstract Human β-defensin-3(HBD3) is a low molecular weight cationic peptide with a broad antimicrobial spectrum. A recombinant *Escherichia coli* (pET32-smHBD3) was constructed to produce HBD3 fusion protein (TrxA-HBD3) before, but the productivity is relatively low. In the present work, the effects of different expression conditions were systematically investigated to improve the expression level of the fusion protein. With regard to the volumetric productivity, the optimal conditions were determined as follows: cultivation at 34 °C in MBL medium, induction at middle stage of the exponential growth phase with 0.4 mM isopropylthio-D-galactoside, and postinduction expression for 8 h. Under these conditions, the volumetric productivity of the fusion protein reached 2.55 g/L, i.e., 0.55 g mature HBD3/L, which was about 2.6 times of that obtained under the unoptimized conditions. And the target protein still maintained high solubility (≥97.9%) and accounted for 66% of the total soluble protein. A cationic exchange purification step was employed to obtain high-purity target protein (90%) with a recovery ratio of 78%. This soluble expression level of HBD3 fusion protein was the highest among all the reported literature and facilitated the development of high efficient purification of HBD3.

Keywords Antimicrobial peptide · Human β-defensin-3 · Optimization · Purification · Soluble expression

Introduction

Defensins, produced by plants, vertebrates, invertebrates, and mammals, are a set of small cysteine-rich antimicrobial peptides with three or four intramolecular cysteine disulfide bonds and with a large β-sheet structure. These peptides are highly effective in killing a wide range of microorganisms and play an important role in the innate immune defense. Due to their unique mechanism in antimicrobial action, defensins are promising antibiotics with low susceptibility to

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acquired bacterial resistance [1]. Differing with respect to the location and connectivity of six cysteine residues, mammal defensins are classified into three subfamilies: the α-, β-, and θ-defensins [2]. To date, at least ten human defensins have been characterized: six α-defensins and four β-defensins. Recently, a large number of putative new human β-defensin genes were reported by using HMMER (a computational search tool based on hidden Markov models) combining with BLAST (basic local alignment search tool) from human genome data bank [3, 4].

Human β-defensin-3(HBD3), a cationic peptide with 45 amino acid residues, was first isolated from human lesional psoriatic scales and cloned from keratinocytes in 2000 [5]. As an antibiotic peptide, HBD3 has a broad antimicrobial activity against Gram-negative and Gram-positive bacteria, and fungi, including *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, multiresistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium* and *Burkholderia cepacia* [5, 6]. Different from other human defensins, which are salt-sensitive in antimicrobial activity, HBD3 maintains its broad spectrum of activity at physiologic saline levels [6–8]. Therefore, HBD3 is an ideal antimicrobial peptide for therapeutic applications and the production of HBD3 with recombinant techniques may accelerate the development of its therapeutic research and mechanism studies.

It is important for the recombinant protein to have an accurately folded form for its full bioactivity, while insoluble aggregates with low renaturation yields, which always occurs when high-level expressions are achieved in *E. coli* [9, 10], are generally misfolded and thus biologically inactive. Thus, soluble expression strategies are preferred to produce functional proteins efficiently, avoiding the refolding procedure. In our previous work, soluble fusion protein containing human β-defensin-2 (HBD2) and HBD3 were successfully expressed after codon optimization in *E. coli* as well as the cell-free system, and the purified mature HBD2 and HBD3 were fully bioactive [11–17]. However, the productivity (0.99 g/L) of HBD3 fusion protein in our constructed recombinant *E. coli* strain [12] was relatively low. Due to the a relatively low recovery rate of mature human beta-defensin 3 after a multi-step down-stream purification process, a high-level expression of this HBD3 fusion protein is preferred for the large-scale preparation of mature recombinant HBD3. Some effective strategies of optimizing expression conditions had been adopted to greatly improve the expression of HBD2 fusion protein in our laboratory [14]. In the present work, we focused on the investigation of the effects of expression conditions on the productivity of HBD3 fusion protein, and the expression level of this target protein was obviously improved after the optimization of these expression conditions. By utilizing the high pI (8.4) of the target protein, one cationic exchange chromatographic process was developed to efficiently purify the HBD3 fusion protein.

Materials and Methods

Strains

A recombinant *E. coli* BL21 (pET32-smHBD3) was used to express the fusion HBD3 (TrxA-HBD3) [12].

Medium

Luria–Bertani (LB) medium (*w*/*v*): 0.5% yeast extract, 1% tryptone, 1% NaCl [18]; 2× YT medium (*w*/*v*): 1.6% tryptone, 1% yeast extract, 0.5% NaCl [14]; MBL medium (*w*/*v*): 0.5% glucose, 3% yeast extract, 2% tryptone, 0.35% $(NH4)_2HPO_4$, 0.35% KH_2PO_4, 0.5% K_2HPO_4, 0.7% $MgSO_47H_2O$, and 2.1%NaCl [14].

Expression and Cell Disruption

A fresh clone of *E. coli* BL21 (DE3), harboring the pET32-smHBD3 vector, was grown in LB medium containing 50 μg ampicillin/mL. When cultured to an optical density (OD_{600}) of 0.4, the cells were inoculated at 5% (*v*/*v*) into MBL medium in 250 mL flask under various cultivation conditions. The protein expression was induced with isopropylthio-D-galactoside (IPTG). After continued culture for a certain time, cells were harvested by centrifugation at 4,000×*g* for 20 min, then resuspended in 20 mM Tris–HCl (pH 7.5), and disrupted by sonication. After the centrifugation at 13,000×*g* for 30 min, the supernatant was examined for soluble protein, and the insoluble portion was further treated by dissolving in 8 M urea.

Purification of Recombinant HBD3 Fusion Protein

The HBD3 fusion protein was purified from the supernatant of culture by cationic exchange chromatography. All the chromatograph operations were performed on the ÄKTA Purifier system (Amersham Pharmacia Biotech, Umeå Plant, Sweden), with CM Sepharose® Fast Flow (Pharmacia Biotech) as the media for the cationic exchanger. The column (XK16 Phamacia) filled with CM Sepharose was initially equilibrated with buffer A (50 mM NaH_2PO_4–Na_2HPO_4, 50 mM NaCl, pH 7.4). The flow rate of soluble protein fraction was 1.0 mL/min. After washing with three bed-volume buffer A, the bound fusion proteins were eluted with buffer B (50 mM NaH_2PO_4–Na_2HPO_4, 1 M NaCl, pH 7.4). The eluate was collected for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Analysis of Proteins

Standard SDS-PAGE method was applied for fusion protein assay. The fraction of target protein was quantified by Quantity One software, Version 4.4.0 (Bio-Rad, USA). Bradford protein assay was used for quantitative analyses of proteins [19].

Results and Discussion

Effects of Different Media on Cell Growth

The medium composition is a main factor of influencing cell growth. And a high cell density is helpful for high-level expression in general. Three kinds of media were examined and the cell growth profiles were compared. The results were shown in Fig. 1. Among three kinds of medium, the highest cell density of BL21 (DE3)/pET32-smHBD3 was obtained in MBL. In accordance with the conclusion in the previous work about HBD2 [14], rich nutrition (MBL) was favorable for cell growth. MBL medium was employed for subsequent experiments.

Effects of Temperature on HBD3 Fusion Protein Expression

Temperature is an important parameter in recombinant protein expression [18]. The cultivation of BL21 (DE3)/pET32-smHBD3 in MBL medium was carried out at various temperatures. As shown in Fig. 2a, the solubility of the fusion protein maintained high from 28 to 34 °C (over 95%), while at 37 °C, the solubility was still high (91.8%), indicating that the solubility of the fusion protein was high at a relatively wide tested temperature range,

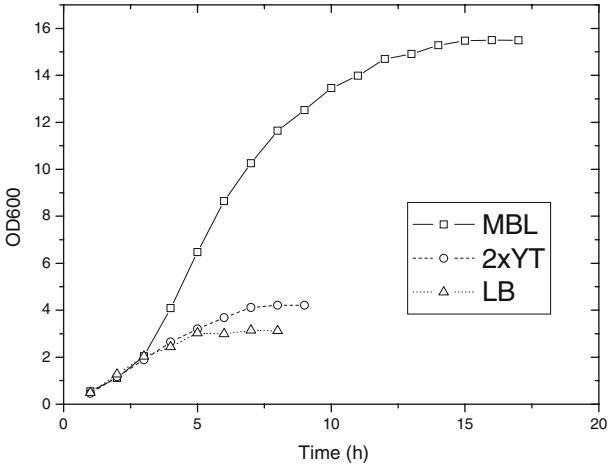

Fig. 1 Growth profiles of BL21 (DE3)/pET32-smHBD3 in MBL, 2× YT, and LB medium

even at 37 °C. The highest productivity (1.41 g/L) of soluble target protein was attained at 34 °C, and the soluble target protein accounted for 61% of total soluble proteins (Fig. 2b). Thus, 34 °C was optimal for the soluble expression of this target fusion protein in this recombinant *E. coli* strain.

Effects of Medium Volume on HBD3 Fusion Protein Expression

Cultivation was carried out in 250 ml shake flasks and the effect of different medium volume on fusion protein expression was evaluated. It was observed in Table 1 that the highest concentrations of total soluble proteins (3.67 g/L) and soluble target fusion product (1.84 g/L) were obtained when initial medium volume was 20 ml. There was one trend that the expression level of soluble target protein increased with the decreasing of medium volume in the shake flask. The results suggested that dissolved oxygen was an important factor for either cell growth or the expression of HBD3 fusion protein. This is accordant with our previous observance about HBD2 and hEGF expression in *E. coli* [14, 20].

Effects of Induction Timing on HBD3 Fusion Protein Expression

Isopropylthio-D-galactoside induction is the start point in the process of the foreign protein expression, which brings great changes to the metabolism of host cells by initiating the transcription of the foreign gene in the plasmid. As shown in the time-course profile of cell growth at 34 °C in MBL medium, the cell density can grow to an OD_{600} value of 16 after about 16 h. The effect of induction timing was evaluated by adding IPTG at different stages of exponential growth phase and harvested at the stationary stage. The soluble fusion protein and the total soluble protein were analyzed (Table 1). At different induction times, the concentration of soluble fusion protein (TrxA-HBD3) varied in a wide range from 1.27 to 2.07 g/L, and the fusion protein percentage varied from 44 to 52%. The highest concentration and the largest percentage were observed when induced at the middle stage of exponential growth phase (OD_{600}=11), which was consistent with our previous observation in the IPTG-induced expression of HBD2 [14]. Apparently, the induction of *E. coli* culture at a relatively high density would benefit the expression of the target protein because IPTG induction at early stage inhibited the growth of recombinant *E. coli* and reduced the

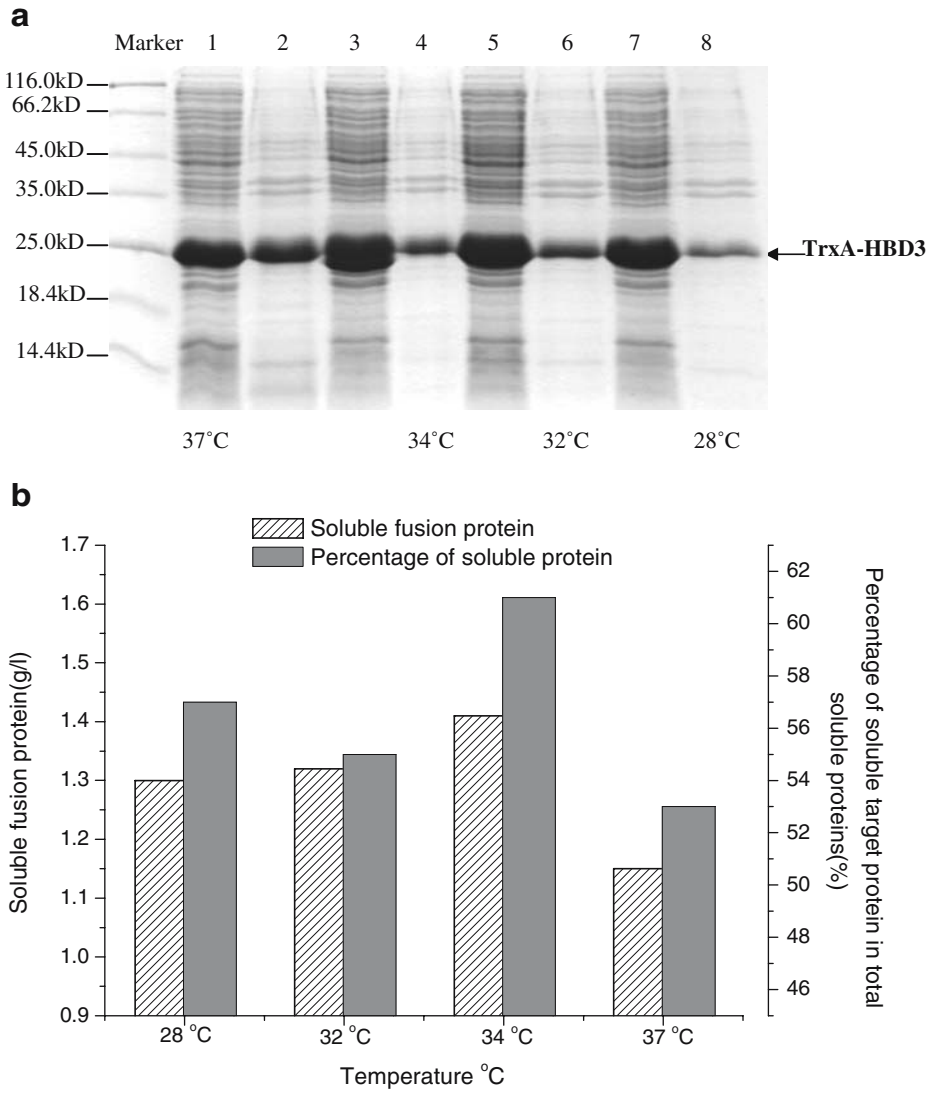

Fig. 2 Effects of different temperatures on the expression of target fusion protein. (**a**) SDS-PAGE analyses of soluble and insoluble proteins with BL21 (DE3)/pET32-smHBD3 at various temperatures. Soluble protein, *lanes 1*, *3*, *5*, and *7*; insoluble protein, *lanes 2*, *4*, *6*, and *8*. (**b**) The solubility of fusion target protein at different temperature, which was calculated as the ratio of band-density of "soluble fusion protein/total fusion protein"

stability of plasmid in the recombinant cells. The induction at the middle stage of exponential growth would balance the cell growth and the efficiency of IPTG induction, and thus was adopted in the succeeding experiments.

Effects of IPTG Concentration on HBD3 Fusion Protein Expression

For the expression vector, pET32-smHBD3, the foreign protein expression was triggered by adding IPTG into the culture medium. The added IPTG concentration should be examined

Table 1 The effects of medium volume, induction timing, and IPTG concentration on the expression of soluble HBD3 fusion protein.

	Medium volume (ml)[a]				Induction timing[b]				IPTG concentration (mM)[c]			
	20	30	40	50	Early	Middle	Late	0.05	0.2	0.4	0.6	0.8
Total soluble protein (g/l)	3.67	3.22	2.62	2.58	2.60	3.98	3.53	3.28	3.68	3.93	3.82	3.72
Fusion/total soluble protein (%)	53	55	52	50	49	52	44	63	60	60	55	53
Fusion protein (g/l)	1.84	1.77	1.36	1.29	1.27	2.07	1.55	2.07	2.21	2.36	2.10	1.97

All tests were carried out in 250 ml flasks at 34 °C, and the postinduction expression time is 8 h.

[a] IPTG was added at the middle stage of exponential growth with final concentration of 0.8 mM.

[b] Medium volume is 30 ml; IPTG was added in a final concentration of 0.8 mM at various stages of exponential growth phase: early (OD_{600} 3), middle (OD_{600} 11), and late (OD_{600} 14).

[c] Medium volume is 30 ml; IPTG was added at the middle stage of exponential growth.

because it significantly affects recombinant protein expression [18, 20]. The foreign protein could not be expressed fully when the IPTG concentration was too low and the high IPTG concentration would be harmful to cell growth [21]. In this work, the IPTG concentration from 0.05 to 0.8 mM was examined and the result was shown in Table 1. Different IPTG concentrations in the tested range had efficient induction effect for the expression of the target protein, and the highest levels of the soluble product and the total soluble protein were achieved simultaneously by adding 0.4 mM IPTG to the culture. This optimal inducer concentration was used for further optimization of the expression conditions.

Effects of Postinduction Time on HBD3 Fusion Protein Expression

The target protein begins to be synthesized after adding the IPTG into the medium. However, the concentration of target protein is not proportional to the expression time. The optimal expression time was determined by analyzing samples taken at 2, 4, 6, 8, 10, and 12 h, after induction in optimized conditions. The productions of soluble and insoluble product were analyzed by SDS-PAGE and compared (Fig. 3 and Table 2). The concentrations of soluble target protein and the total soluble protein both reached the maximum of 2.55 and 3.86 g/L, respectively, after expression for 8 h, with the highest percentage of target protein in the total soluble proteins of 66%. The result also showed that the insoluble target protein increased with expression time. In addition, soluble product decreased after 8 h. In overtime expression, some soluble fusion protein may be

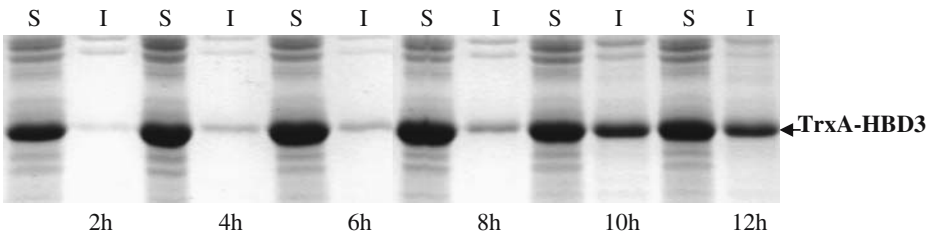

Fig. 3 Effects of different postinduction times on the expression of target fusion protein with BL21 (DE3)/pET32-smHBD3. Soluble and insoluble proteins were analyzed at various postinduction times by using SDS-PAGE. *S*, soluble fractions; *I*, insoluble fractions

Table 2 The effects of postinduction time on the expression of soluble HBD3 fusion protein.

	Postinduction time (h)					
	2	4	6	8	10	12
Total soluble protein (g/l)	3.17	3.42	3.56	3.86	3.79	3.69
Fusion/total soluble protein (%)	52	58	63	66	58	59
Fusion soluble protein (g/l)	1.65	1.98	2.24	2.55	2.20	2.18
Fusion insoluble protein (mg/l)	5.0	31.1	41.1	53.4	126.3	158.0
Solubility (%)	99.7	98.5	98.2	97.9	94.5	93.2

Test was carried out in 250 ml flasks at 34 °C, medium volume is 30 ml, and IPTG was added at the middle stage of exponential growth with final concentration of 0.4 mM.

transformed into insoluble product, or the target protein was produced in insoluble form. Thus, one optimal expression was 8 h with regard to volumetric yield of HBD3.

Primary Purification of HBD3 Fusion Protein

In our previous work, HBD3 fusion protein was efficiently purified by nickel-affinity chromatography [12]. Because the pI of HBD3 fusion protein is 8.4, cationic exchange

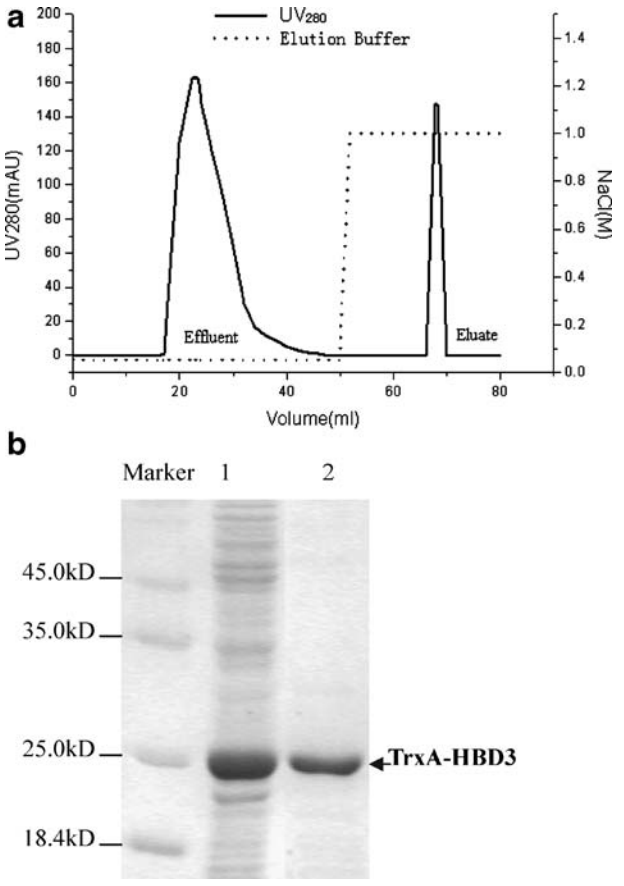

Fig. 4 Primary purification of HBD3 fusion protein using cationic exchange chromatography. (**a**) The curve of cationic exchange chromatography. (**b**) SDS-PAGE analyses of collected samples of cationic exchange chromatography. *Lane 1*, loaded sample; *lane 2*, the purified HBD3 fusion protein

chromatography was employed to purify the target protein in the present work. The cationic exchange chromatography curve of UV_{280nm} was shown in Fig. 4a. After the binding of the soluble protein sample on CM Sepharose® Fast Flow resin, 1 M NaCl buffer (pH 7.4) was used to elute the target cationic fusion protein effectively. After SDS-PAGE analyses of the elution fractions, the peak of eluate was collected as the purified sample of HBD3 fusion protein with a high purity of 90%. The recovery rate of this fusion protein in this primary purification process was 78%. Although nickel affinity chromatography could get a little higher purity (95%) and recovery rate (84%) [12], cationic exchange chromatography has a lower operation cost and could be easily scaled up. Therefore, cationic exchange chromatography was an efficient alternative method for purifying HBD3 fusion protein.

Conclusion

A relatively low productivity of HBD3 fusion protein (0.99 g/L) was produced with recombinant *E. coli* BL21 (pET32-smHBD3) under the unoptimized conditions before. After the optimization of some expression conditions, such as medium composition, temperature, and induction conditions, the volumetric productivity of the soluble fusion protein (TrxA-HBD3) was improved to 2.55 g/L. And the target protein still maintained high solubility (≥97.9%) and accounted for 66% of the total soluble protein. This soluble expression level of HBD3 fusion protein was the highest among all the reported literature. After a single step of cationic exchange chromatography, the purified HBD3 fusion protein was obtained with a recovery ratio of 78%. According to our previous work, HBD3 would be bioactive when it was cleaved from soluble fusion protein with TrxA as a fusion partner [12]. Therefore, this high-level soluble expression strategy was favorable for obtaining bioactive HBD3 later by enzymatic cleaving of the fusion protein and down-stream purification processes. The present work would be helpful to the large-scale production of this and other similar peptides.

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