African Journal of Biotechnology Vol. 11(39), pp. 9479-9484, 15 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.162
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Expression of human β-defensin-1 in recombinant *Escherichia coli* and analysis of its antimicrobial spectrum

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Accepted 25 April, 2012

***Escherichia coli* BL21(DE3) was transformed with a pHCD1 plasmid harboring the human β-defensin-1 (*hBD1*) gene fused in frame behind a disulfide bond isomerase (DsbC), a His-tag, and an enterokinase cleavage site. After induction, the DsbC-hBD1 was expressed as a ~36 kDa soluble fusion protein in recombinant *E. coli*, which also inhibited host cell growth. After cell disruption, the soluble protein was easily recovered by Ni^{2+} affinity chromatography and cleaved by enterokinase to yield a mature hBD1 of about 4 kDa. Importantly, the mature hBD1 showed broad antimicrobial activity against Gram-positive and -negative pathogenic bacteria, including *Streptococcus pneumoniae*, *E. coli* O157:H7, and *Klebsiella pneumoniae*.**

Key words: Antimicrobial activity, *Escherichia coli*, human β-defensin-1, soluble expression.

INTRODUCTION

Defensins are members of a family of small cationic antimicrobial peptides present in mammalian epithelial cells and phagocytes, contributing to host mucosal and epithelial defense in the small intestine and skin, respectively. They show a broad range of antibacterial activity against Gram-positive and -negative bacteria, fungi, and enveloped viruses (Ganz, 2003). There are three subfamilies, α-, β- and θ-defensins, classified according to the spatial distribution of the cysteine residues as one of structural characteristics (Tang et al., 1999). To date, several human α-defensins (neutrophil defensins; HNP1 to HNP4 and other α-defensins; hD5 and hD6) and human β-defensin (hBD1 to hBD6 and Conejo et al., 2001; Harder et al., 2001). The gene encoding hBD1 was originally purified from the plasma of patients with end-stage renal disease and is located hBD25 to hBD29) have been discovered (Jones and Bevins, 1993; Wilde et al., 1989; Bensch et al., 1995; within a cluster on chromosome 8p23 (Liu et al., 1997). Unlike inducible hBD members (hBD2 to hBD4), hBD1 is constitutively expressed in epithelial cells of the prostate, kidney, pancreas, trachea and gastrointestinal tract (Bensch et al., 1995). hBD1 is insensitive to low pH, but sensitive to salt, that is, the antimicrobial activity of hBD1 is inhibited by high salt levels (Valore et al., 1998).

Over the past decade, recombinant baculovirus expression and chemical synthetic method have been used to produce hBDs as potential therapeutic agents (Harder et al., 2001; Valore et al., 1998). Since then, several hBDs have been prepared from recombinant *Escherichia coli* cells, which grow rapidly and express proteins easily (Huang et al., 2008, 2009; Si et al., 2007; Wang et al., 2004). Specific strategies such as fusion proteins or codon optimization have been employed in

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Abbreviations: hBD1, Human β-defensin-1; **DsbC,** disulfide bond isomerase.

these recombinant *E. coli* systems to prevent cytotoxicity and sensitivity to proteolytic degradation of the expressed hBDs (Li et al., 2004; Piers et al., 1993). However, these strategies have sometimes led to the formation of inclusion bodies and biological inactivation. Therefore, it would be beneficial to develop ways to produce a soluble form of bioactive hBD by genetic engineering.

In a previous study, we constructed a recombinant plasmid, pOED1, harboring the hBD1 gene fused in frame behind a disulfide bond isomerase (DsbC) as fusion protein, and introduced the plasmid into *Lactococcus lactis* producing bacteriocin. The results show that recombinant *L. lactis* produced bioactive hBD1 with bactericidal activity against *E. coli* JM109 (Choi et al., 2005). In the present study, the plasmid pHCD1 constructed previously, was used to express the DsbC-hBD1 fusion protein which was directly expressed as a soluble fusion protein in recombinant *E. coli* BL21(DE3). We also purified the fusion protein and cleaved to obtain mature hBD1 and examined its spectrum of antimicrobial activity in this study.

MATERIALS AMD METHODS

Expression of DsbC-hBD1 in recombinant *E. coli*

Plasmid pHCD1 harboring the DsbC-hBD1 gene, previously cloned into pET-40b(+) (Choi et al., 2005), was transformed into *E. coli* BL21(DE3). Luria Bertani (LB) agar plates containing 30 µg/ml kanamycin were then used to select for positive clones harboring pHCD1. Recombinant *E. coli* harboring pHCD1 were cultivated at 37°C in 500 ml of LB broth supplemented with kanamycin. After cultivation for 3 h, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added with a final concentration of 1 mM and the culture was incubated for a further 5 h. Cell growth was determined by monitoring the optical density at 600 nm (OD_{600}) and cell pellets were obtained by centrifugation at 12,000 rpm for 20 min.

Purification and preparation of mature hBD1

Pelleted *E. coli* harboring pHCD1 were resuspended in lysis buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 10 mM imidazole, pH 7.4). After disruption by sonication, the cell debris was removed by centrifugation at 12,000 rpm for 20 min to collect the cell-free extract which was applied to a Ni^{2+} affinity column (Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated with lysis buffer and washed with 20 mM imidazole in lysis buffer. The fusion proteins were then eluted with an elution buffer (300 mM Na_2HPO_4, 0.5 M NaCl, 250 mM imidazole, pH 7.4). After collecting the eluted proteins, the inherent buffer in the sample was exchanged for an enterokinase buffer (50 mM Tris-HCl, 1 mM $CaCl_2$, 0.1% Tween-20, pH 8.0) via dialysis. The fusion proteins were then specifically digested with an enterokinase (EKMax™, Invitrogen, Carlsbad, CA, USA), followed by removal of enterokinase using EKapture agarose (Novagen, San Diego, CA, USA) according to the manufacturer's instructions. The digested mature hBD1 was desalted by dialysis against 10 mM sodium phosphate buffer (pH 7.4) and used directly for the antimicrobial activity assays.

Western blot analysis

Western blot analysis was performed as described previously (Choi et al., 2005). Briefly, the proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane using Mini-Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA). After washing the membrane with 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 0.05% Tween-20, the membrane was blocked with 5% skim milk, incubated with Penta-His antibody (Qiagen), followed by alkaline phosphatase-conjugated Anti-Mouse IgG (Sigma), according to the manufacturer's protocol. The specific protein band was then visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate kit (Sigma).

Antimicrobial activity of mature hBD1

Antimicrobial activity was evaluated using the colony forming unit (CFU) assay (Raj et al., 2000). The indicator strains listed in Table 1 were grown in fresh culture medium to a concentration of 10^8 CFU/ml, harvested, and then washed twice in 10 mM sodium phosphate buffer (pH 7.4). Subsequently, the washed cells were diluted to 10^5 CFU/ml in sodium phosphate buffer. The mature hBD1 protein (20 µg/ml) was added to 100 µl of each cell suspension in a total volume of 200 µl. As a control, sodium phosphate buffer was used instead of mature hBD1. After incubation at the corresponding temperatures listed in Table 1 for 2 h, each mixture was serially diluted with the same buffer and then plated on the respective agar plates. The plates were incubated at the respective temperatures for 24 h and the resulting colonies counted. Finally, antimicrobial activity was determined using the following equation: [1-(number of viable colonies after incubation with the mature hBD1)/(number of viable colonies after incubation with buffer alone)]×100, which represents the loss of cell viability.

RESULTS AND DISCUSSION

Plasmid pHCD1 was previously constructed by fusing the hBD1 gene with DsbC, a his-tag, and an enterokinase cleavage site in a pET-40b(+) vector (Choi et al., 2005). The DsbC protein encodes a periplasmic protein with disulfide isomerase activity, which enhances protein solubility and proper folding (Kurokawa et al., 2000). Also, the presence of the his-tag and the enterokinase cleavage site upstream of hBD1 facilitated the purification of DsbC-hBD1 by Ni^{2+} affinity chromatography and efficient enzymatic cleavage of DsbC-hBD1, respectively (Choi et al., 2005). After induction of recombinant *E. coli*, SDS-PAGE analysis of the cell-free extract revealed that the molecular weight of the hBD1 fusion protein was about 36 kDa that was identical to the predicted molecular weight of the recombinant protein; 32 kDa for the DsbC fusion tag upstream of hBD1 and 4 kDa for the mature hBD1 (Figure 1A). This fusion protein (1.1 g/l) was highly soluble (95% solubility) and accounted for 46% of total soluble proteins. This predicted band was also verified by western blot analysis, where a single band was detected in the cell-free extract from recombinant *E. coli* harboring pHCD1, suggesting that the DsbC-hBD1 fusion protein was expressed in a soluble form (Figure 1B). There have been several studies reporting the expression of hBDs in *E. coli* using several fusion partners, including glutathione S-transferase (GST) (Si et al., 2007; Wang et al., 2004) and

Table 1. Indicator strains used in this study and the antimicrobial spectrum of mature hBD1.

Indicator strain[1]	Source	Medium[2]	Temperature (°C)	Antimicrobial activity (%)[5]
Acinetobacter calcoaceticus	KCCM 40204	NB	30	−
Acinetobacter sp.	KCCM 11771	NB	37	−
Actinomyces viscosus	KCCM 12074	BHI	37	+++ (91)
Aeromonas hydrophila	KCCM 11533	NB	30	+++ (90)
Aeromonas salmonicida	KCCM 40239	BHI	26	+++ (94)
Aeromonas sobria	KCCM 41093	TSB	30	+++ (98)
Bacillus brevis	KCCM 11711	NB	30	+ (30)
Bacillus cereus	KCCM 11204	NB	30	−
Bacillus circulans	KCCM 11685	TSB	30	+++ (97)
Bacillus coagulans	KCCM 11712	NB	37	+++ (95)
Bacillus licheniformis	KCCM 11237	NB	30	+++ (97)
Bacillus longisporus	KCCM 41413	NB	30	−
Bacillus megaterium	KCCM 11492	NB	30	+++ (99)
Bacillus pumilus	KCCM 11874	NB	30	−
Bacillus sphaericus	KCCM 35419	BP[3]	37	+ (35)
Bacillus subtilis	KCCM 11314	NB	30	+++ (97)
Bacillus thuringiensis	KCCM 11428	NB	30	+++ (96)
Candida albicans	KCCM 11282	YM	25	+++ (96)
Citrobacter freundii	KCCM 11931	NB	37	−
Corynebacterium diphtheriae	KCCM 40413	BHI	37	+++ (95)
Cryptococcus neoformans	KCCM 50544	YM	26	−
Enterobacter aerogenes	KCCM 40146	NB	37	+++ (98)
Enterobacter cloacae	KCCM 11909	NB	30	+ (32)
Erwinia chrysanthemi	KCCM 11653	NB	30	+++ (95)
Escherichia coli JM109	ATCC 53323	LB	37	+++ (99)
Escherichia coli O157:H7	ATCC 35150	LB	37	++ (87)
Klebsiella oxytoca	KCCM 41043	NB	37	+++ (98)
Klebsiella pneumoniae	KCCM 11418	NB	37	+++ (95)
Listeria monocytogenes	ATCC 15313	BHI	37	−
Neisseria mucosa	KCCM 11703	NB	37	+++ (96)
Plesiomonas shigelloides	KCCM 40891	TSB	37	+++ (96)
Proteus mirabilis	KCCM 11381	NB	37	+++ (96)
Proteus vulgaris	KCCM 11539	BPS[4]	30	+ (46)
Providencia alcalifaciens	KCCM 40889	TSB	37	+++ (92)
Providencia rettgeri	KCCM 11799	NB	37	+++ (98)
Pseudomonas aeruginosa	KCCM 11540	NB	37	+ (47)
Salmonella choleraesuis	KCCM 11863	NB	37	++ (62)
Salmonella typhimurium	KCCM 40253	NB	30	+++ (99)
Serratia marcescens	KCCM 11808	NB	26	+++ (98)
Shigella sonnei	KCCM 11903	NB	37	+++ (91)
Streptococcus agalactiae	KCCM 11957	BHI	37	+ (12)
Streptococcus gordonii	KCCM 35495	BHI	37	−
Streptococcus mutans	KCCM 40105	BHI	37	−
Streptococcus pneumoniae	KCCM 40410	BHI	37	+++ (97)
Streptococcus pyogenes	KCCM 11817	BHI	37	+++ (91)
Vibrio alginolyticus	KCCM 40513	M	37	−
Vibrio carchariae	KCCM 40865	M	26	−
Vibrio mimicus	KCCM 40826	NB	37	+++ (92)
Vibrio parahaemolyticus	ATCC 17802	NB with 3% NaCl	37	+ (20)

[1]The indicator strains were obtained from the Korean Culture Center of Microorganisms (KCCM) and American Type Culture Collection (ATCC). [2]All culture medium containing nutrient broth (NB), brain heart infusion (BHI), trypticase soy broth (TSB), yeast malt broth (YM), Luria-Bertani broth (LB), and marine broth (M) were purchased from Difco/BBL. [3]Beef extract broth (BP) medium contains 1% beef extract, 1% peptone and 0.5% NaCl (w/v). [4]Beef extract-peptone-serum broth (BPS) medium contains 1% beef extract, 1% peptone, 0.1% NaCl (w/v) and 5% bovine serum (v/v). [5]Antimicrobial activity was reflected by the loss of cell viability (%) of each indicator strain induced by mature hBD1; −, no inhibition; +, <50%; ++, 50 to 90%; +++, >90%.

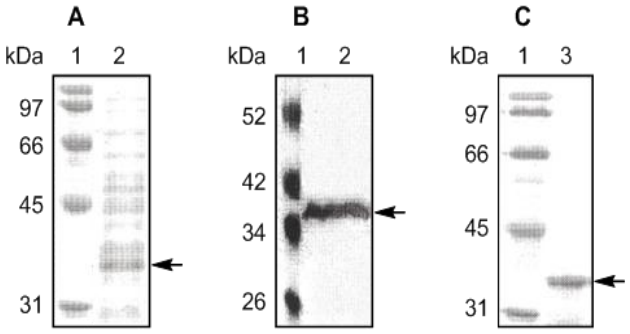

Figure 1. SDS-PAGE (A) and western blot analysis (B) of the hBD1 protein fused with DsbC and SDS-PAGE analysis (C) of the DsbC-hBD1 fusion protein purified by Ni^{2+} affinity chromatography. Lane 1, protein molecular weight markers; lane 2, cell-free extract of recombinant *E. coli* BL21(DE3) harboring pHCD1; lane 3, purified DsbC-hBD1 fusion protein. The solid arrow indicates the DsbC-hBD1 fusion protein.

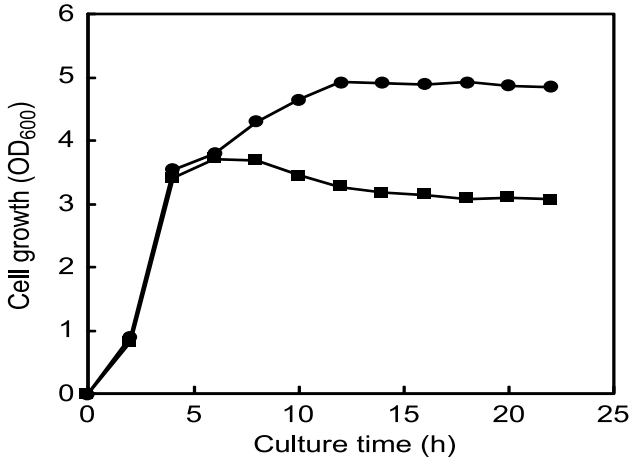

Figure 2. Effect of DsbC-hBD1 fusion protein expression on the growth of host *E. coli* BL21(DE3) cells. Closed circles and squares indicate recombinant *E. coli* BL21(DE3) harboring pHCD1 plasmid not induced and induced by IPTG after the cultivation for 3 h, respectively.

thioredoxin (Huang et al., 2008, 2009).

However, few studies have used a DsbC fusion protein to express hBDs and showed a soluble expression of hBD1 in *E. coli*. Therefore, these results could be meaningful, showing that fusing a target protein with DsbC protein is a powerful and promising strategy for producing soluble hBD1 in *E. coli*. DsbC is required for disulfide bond formation during oxidative protein folding in the bacterial periplasm (Hiniker et al., 2005). Therefore, fusing DsbC to a target hBD1 could facilitate the accurate folding of disulfide bonds and, thereby, the expression of soluble protein.

Based on the fact that hBD1 exhibited the bactericidal activity against *E. coli* JM109 (Choi et al., 2005), it was crucial to investigate the effects of DsbC-hBD1 fusion protein expression on the growth of the host *E. coli* cells. Compared with the control cells with no induction, the growth rate of recombinant *E. coli* did not increase after the induction of hBD1 expression by IPTG (Figure 2). However, cell growth did not seem to be significantly inhibited after IPTG-induction. Therefore, we propose that DsbC prevents hBD1 from extremely exhibiting antimicrobial activity, thereby, promoting the survival of the host cells, which was comparable to the growth inhibition of host cells by expression of GST-hBD3 (Si et al., 2007).

Ni^{2+} affinity chromatography was used to successfully purify the DsbC-hBD1 fusion protein via the His-tag inserted upstream of hBD1 in the pHCD1 plasmid (Figure 1C). The active fraction eluted with 250 mM imidazole (80% product recovery) cleaved by enterokinase to recover mature hBD1 (71% recovery). The purified mature hBD1 was then desalted by dialysis, showing its overall recovery of 63%, and stored for the antimicrobial activity assay. N-Terminal amino acid sequencing analysis of the mature hBD1 indicated that the N-terminal amino acid sequences were DHYNC, which were identical to those of natural hBD1.

CFU assay incorporating various indicator strains was then used to evaluate the antimicrobial activity of mature hBD1 (Raj et al., 2000). As shown in Table 1, mature hBD1 showed a broad range of antimicrobial activity against various pathogenic bacteria. hBD1 showed strong activity against Gram-positive bacteria such as *Bacillus subtilis*, *Corynebacterium diphtheriae* and *Streptococcus pneumoniae,* and Gram-negative bacteria such as *Enterobacter aerogenes*, *Salmonella typhimurium*, and *Vibrio mimicus*. Additionally, hBD1-mediated antimicrobial activity against *E. coli* and *Pseudomonas aeruginosa* was consistent with those shown by other hBDs, including hBD2 (Wang et al., 2004), hBD3 (Huang et al., 2006), hBD4 (Conejo et al., 2001), and hBD28 (Schulz et al., 2005). Comparing with antimicrobial activities against just *E. coli* as indicator strain by other recombinant hBDs produced from recombinant *E. coli* systems, the survivability of *E. coli* JM109 was below 2% at the concentration of 2 μg/ml hBD1 (data not shown). On the other hand, 95% of *E. coli* K12 D31 cells were inactivated in the presence of 50 μg/ml hBD3 (Huang et al., 2006) and 10 μg/ml hBD4 (Xu et al., 2006). *E. coli* K12 cells were also suppressed below 5% viability when 15 μg/ml hBD5 and hBD6 (Huang et al., 2008), and 20 μg/ml hBD26 and hBD27 (Huang et al., 2009) were used. The growth inhibition of *Candida albicans* by hBD1 was also similar to those of hBD2 and hBD3 (Harder et al., 2001) as well as HNP1 and HNP2 (Raj et al., 2000), whereas inhibition of *Klebsiella pneumoniae* was the same as those of hBD2, hBD3, and hBD28. hBD2, hBD3, hBD4, and hBD28 were reported to show antimicrobial activity against *S. pneumoniae*, which is consistent with the results of the present study of hBD1. Bioactivity against *Streptococcus pyrogenes* was also detected, similar to

that of hBD3. However, like HNP1, HNP2 and HNP3, hBD1 did not exhibit antimicrobial activity against *Streptococcus gordonii* and *Streptococcus mutans*. Also, hBD1 did not display antimicrobial activity against *Listeria monocytogenes*, whereas the hD5 protein did (Hsu et al., 2009). The difference in the antimicrobial spectrum exhibited by the different hBDs and HNPs is still not fully understood, and would benefit from future study (Huang et al., 2009).

hBD1 and hBD2 have been known to be particularly effective against Gram-negative bacteria, but relatively less potent against Gram-positive bacteria, whereas hBD3 is effective against a broad range of Gram-positive and Gram-negative bacteria (Pazgier et al., 2006). However, an interesting study recently reported that the structure of hBD1 under reduced environment could be changed and therefore free cysteins in the carboxy terminus seem to be effective to inactivate the Gram-positive bacteria as equal as hBD3 (Schroeder et al., 2011). In fact, it could be hard to say that the recombinant hBD1 produced in this study is the reduced form of hBD1 and it did not inactivate the growth of all indicator strains including Gram-positive and -negative bacteria. However, it could be possible to note that the hBD1 in this study seems to be a potential bioactive agent with a broad antimicrobial spectrum, because it exhibited the growth-inactivation of some Gram-positive bacteria as well as Gram-negative bacteria, unlike other hBD1 previously reported. To prove this suggestion, the antimicrobial activity of recombinant hBD1 should be conducted dependent on redox modulation of hBD1 in future studies (Schroeder et al., 2011). Additionally, the expanded antimicrobial spectrum of hBD1 (and/or reduced hBD1) should be investigated using a variety of fungi and viruses, since the antimicrobial spectrum exhibited by the same hBD might vary due to different indicator strains and research areas (Si et al., 2007).

Although we described the analysis of antimicrobial spectrum of hBD1 expressed and purified from recombinant *E. coli* BL21(DE3), it should be noted that the antimicrobial activity is not absolutely dependent on right-folding hBD. The antimicrobial activity could remain regardless of native disulfide-linked cysteines of hBDs (Wu et al., 2003), whereas the chemoattraction of macrophages could be more conserved with them (Soruri et al., 2007), suggesting that the immunomodulatory properties of recombinant hBD1 is need to be characterized in future work.

In summary, hBD1 was successfully expressed as a soluble fusion protein (DsbC-hBD1) in recombinant *E. coli* BL21(DE3). After expression, DsbC-hBD1 was easily recovered via one-step purification on a Ni^{2+} affinity column, and the mature hBD1 subsequently released from the fusion partner by enterokinase cleavage. Also,an antimicrobial activity assay incorporating mature hBD1 showed that hBD1 has broad antimicrobial activity against both Gram-positive and -negative pathogenic bacteria. This study contributes to the greater understanding of the hBD1 antimicrobial spectrum and suggests that hBD1 may be a potential therapeutic agent which can be developed by the pharmaceutical industry.

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