J Ind Microbiol Biotechnol (2012) 39:11–17
DOI 10.1007/s10295-011-0992-x

ORIGINAL PAPER

Production of bioactive sheep β-defensin-1 in *Pichia pastoris*

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Received: 28 February 2011 / Accepted: 12 May 2011 / Published online: 4 June 2011
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Abstract Previous research has shown that sheep β-defensin-1 (sBD-1), a small cationic peptide with a broad range of antimicrobial activities, could inhibit the growth of both Gram-positive and Gram-negative bacteria as well as that of fungi. In order to increase the yield of current ovine defensin purification methods, mature sBD-1 (msBD-1) was added with a 6-His tag on the C-terminus (msBD-1-T) and expressed in *Pichia pastoris* in the presented work. The msBD-1 and msBD-1-T were expressed in the *Pichia pastoris.* Both msBD-1 and msBD-1-T were purification, and the two peptides were used to inhibit *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Shigella flexneri.* The antimicrobial activity of the 6-His tagged msBD-1-T peptide was not significantly different from that of the native msBD-1 peptide. The two peptides could inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Shigella flexneri* with equal efficiency as well as chemoattractant function. In addition, the yield of purified 6-His-tagged msBD-1 was greater than that of msBD-1. The presented method might be a more efficient approach to produce bioactive sBD-1.

Keywords Bioactive · *Pichia pastoris* · Sheep β-defensin-1

Electronic supplementary material The online version of this article (doi:10.1007/s10295-011-0992-x) contains supplementary material, which is available to authorized users.

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Introduction

Defensins are a well-characterized family of antimicrobial peptides that are divided into three main classes, α-, β-, and θ-defensins, according to the pairing pattern of disulfide bonds among six typical cysteine residues [6, 8]. β-Defensins have been found in many animal species, including bovine, ovine, porcine, and humans [9]. β-Defensins are principally produced by epithelial cells in various organs, including the skin, lungs, kidneys, pancreas, uterus, eyes, nasal mucosa, and oral mucosa [2]. Defensins have also been shown to play a role in both adaptive and innate immunity.

Sheep β-defensin-1 (sBD-1) is an arginine-rich small cationic peptide whose gene encodes 38 amino acid residues. It is approximately 3.8 kDa in size with six cysteines forming three intramolecular disulfide bonds [13]. The antimicrobial action of sBD-1 has been related to its high net charge (+11) coupled with its hydrophobicity [17]. Like human β-defensin, sBD-1 has a broad spectrum of antimicrobial activity in vitro against a variety of bacteria, fungi, parasites, and some viruses. sBD-1 kills bacteria by binding electrostatically to the negatively charged membranes and forming pores within the bacterial cell membrane, resulting in cell lysis [16].

sBD-1 is stored as a propeptide in secretory vesicles and then further processed into a mature bioactive peptide prior to release into the intestinal lumen [7], although the exact mechanism involved in this process is still unclear.

Antimicrobial peptides with short amino acid sequences can be synthesized by chemical methods [9, 19]. However, due to the high cost of chemical peptide synthesis, recombinant approaches using microorganisms as host cells to express these short peptides may be a more efficient alternative method [4]. The methylotrophic yeast *Pichia pastoris* is an effective system for expression and

biosynthesis of recombinant proteins, including defensins. Indeed, previous study reported that hBD-2 could be expressed in *P. pastoris* [5]. However, yields of these methods were relatively low, possibly due to inefficient purification [20].

In order to increase the yield of recombinant sBD-1, mature sBD-1 (msBD-1) was added with a 6-His tag on the C-terminus (msBD-1-T) and expressed in *P. pastoris* in the presented study. Furthermore, expression levels, purification yields, and antimicrobial bioactivity of the recombinant msBD-1-T peptide were determined and compared with the native msBD-1 peptide.

Materials and methods

Strains, plasmids, and culture medium

Escherichia coli (*E. coli*) DH5αF′ (TaKaRa, Japan) was cultured at 37°C in Luria–Bertani (LB) medium. *P. pastoris* was used as the host for the expression of heterologous protein. *E. coli* CMCC 44101, *Staphylococcus aureus* (*S. aureus*) CMCC 26003, *Proteus vulgaris* (*P. vulgaris*) CMCC 49001, *Pseudomonas aeruginosa* (*P. aeruginosa*) CMCC 10102, and *Shigella flexneri* (*S. flexneri*) CMCC 51285 (purchased from the China Medical Culture Collection, Beijing, China) were used for the antimicrobial assays.

Plasmid pMD19-T Simple (TaKaRa, Japan) and Plasmid pPIC9 K (Invitrogen, USA) were used as cloning and expression vectors, respectively. All restriction enzymes and T_4 DNA ligase were purchased from TaKaRa (Japan). LB medium containing (w/v) 0.5% yeast extract, 1% tryptone, and 1% NaCl were used for the manipulation of molecular clones [5]. Buffered glycerol complex (BMGY) [BMGY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 4×10^{-5}% biotin, and 1% glycerol], buffered methanol complex (BMMY) (BMMY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 4×10^{-5}% biotin, and 0.5% methanol), minimal dextrose (MD) (MD; 1.34% YNB, 4×10^{-5}% biotin, and 0.5% glycerol), and minimal methanol (MM) (MM; 1.34% YNB, 4×10^{-5}% biotin, and 0.5% methanol) media were prepared according to the manual of the *Pichia* expression kit (Invitrogen), except that potassium phosphate was omitted from the medium.

Cloning of sBD-1

Sheep small intestinal total RNA was isolated using Trizol reagent (TaKaRa, Japan) according to the manufacturer's instructions, and treated with RNase-free DNase I (TaKaRa, Japan). The concentration and purity of the isolated RNA were determined by monitoring absorbance at 260- and 280-nm wavelength.

The cDNA fragment encoding the sBD-1 protein was amplified by reverse transcription-PCR (RT–PCR) of small intestinal total RNA using *ExTaq* DNA polymerase (TaKaRa, Japan) and the synthetic oligonucleotide primers sBD-1-*Eco*RIF (5′-GAATTCAACATGAGGCTCCATCACCTG-3′) and sBD-1-*Not*IR (5′-ATGCGGCCGCTTACTTCTTTCTGCAGCAT-3′). DNA amplification was conducted with 35 cycles of denaturation (1 min at 94°C), followed by annealing (1 min at 59°C), and elongation (2 min at 72°C). After PCR, the product was cloned into the PMD19-T Simple vector (TaKaRa, Japan) and sequenced. The resulting cloned plasmid was named PMD19-T-sBD-1.

Construction of expression vectors

To construct the *P. pastoris* expression vector, DNA containing sBD-1 but lacking the signal peptide coding sequence was amplified using primers msBD-1F (CCGGAATTCCAATCGRCTAAGCTGCCAT) and msBD-1R (ATTTGCGGCCGCTTACTTCTTTCTGCAGCAT). Amplified DNA was digested with *Eco*RI and *Not*I, and cloned into the pPIC9 K in-frame fusion of the a-factor signal peptide. The resulting construct was designated pPIC9 K-msBD-1. Using a similar strategy, the recombination vector pPIC9 K-msBD-1-T that contained the 6-His tag in the C-terminal region was also obtained. The primer msBD-1-TF was CCGGAATTCCAATCGRCTAAGCTGCCAT, and primer msBD-1-TR was ATTTGCGGCCGCTTAATGATGATGATGATGATGCTTCTTTCTGCAGCAT.

Expression of msBD-1 and msBD-1-T in *P. pastoris*

The two recombination vectors, pPIC9 K-msBD-1 and pPIC9 K-msBD-1-T, were linearized using SacI, and then transferred into *P. pastoris* GS115-competent cells by electroporation (Bio-Rad; conditions: 1.5 kV, 200 Ω, and 25 μF) [14]. The transformed cells were plated on MD plates and incubated at 29°C for 2 or 3 days until colonies appeared. Selected HIS + transformants were transferred to MM and MD plates, and further grown for 2 days at 30°C until colonies formed [18]. The colonies that were normal in MD but very small in MM were selected. These colonies were the HIS+. Then, HIS + transformants were cultivated at 29°C in 5 ml BMGY medium for 48 h (OD600 = 2–6), after which cells were centrifuged and resuspended in 1 ml BMMY containing 0.5% methanol, transformed into 25 ml BMMY medium containing 0.5% methanol in a 250-ml flask and then incubated at 30°C for cultivation at certain conditions according to the optimization purpose [11]. To continue induction, methanol was added to a final concentration of 0.5% every 24 h [3]. Then, after centrifugation at

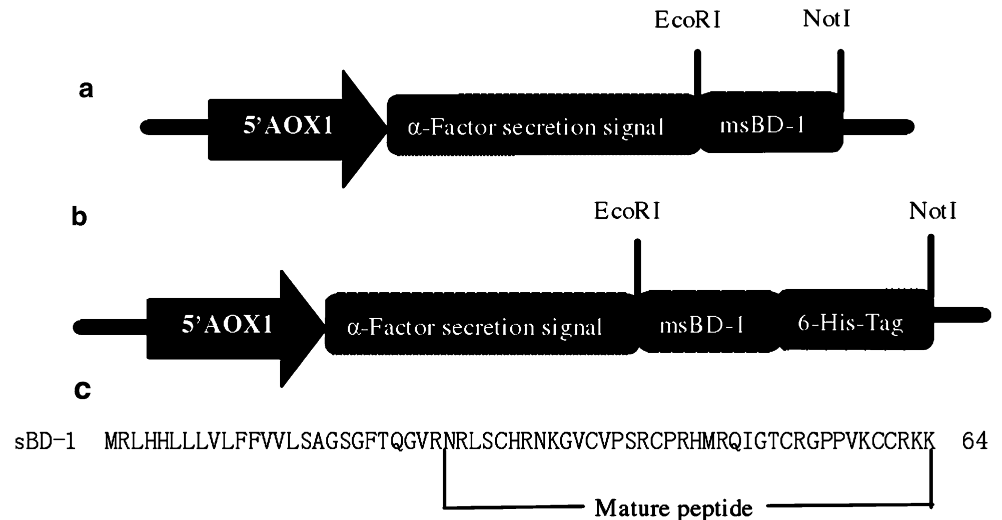

Fig. 1 **a** Schematic of pPIC9 K/msBD-1 expression vectors. The recombinant gene encoding msBD-1 was inserted into the pPIC9 K vector along with the open reading frame of the α-factor signal under the control of the *AOX*1 promoter. **b** Schematic of pPIC9 K/msBD-1-T expression vectors. The recombinant gene encoding msBD-1 and 6-His-tag was inserted into the pPIC9 K vector along with the open reading frame of the α-factor signal under the control of the *AOX*1 promoter. **c** sBD-1 amino acid sequence whose mature peptide contains 38 amino acid

12,000 × *g* for 1 min, the supernatant was collected and subject to analysis using Tricine-SDS–PAGE [22].

Purification of msBD-1 and msBD-1-T

The culture medium after fermentation was first precipitated by being centrifuged at 12,000 × *g* for 15 min at 4°C. The desired proteins were in the supernatant, which was dialyzed against PBS (pH 7.0) for 24 h at 4°C. Because msBD-1 is a cationic peptide, the supernatant was purified using a SP-SEPHADEX (C-25) setup (Amersham Pharmacia). The column filled with positive ion agarose was pre-equilibrated with binding buffer (PBS, pH 7.0) at 1 ml/min. After supernatant loading at 0.5 ml/min and equilibration, the positive ion peptide was eluted using PBS (from pH 7.0 to 3.0 step by 0.2 each elution 100 ml) at 0.5 ml/min, and the material indicated by each absorption peak was collected and then desalted (20 mM Tris, pH 7.8, 50 mM CH_3COONH_4) at 0.25 ml/min by Sephadex G-10 (Amersham Pharmacia), the salt fist outflow, then the objective protein outflow, and lyophilized to a the powder product.

The msBD-1-T peptide containing the 6-His tag was purified by HisTrap FF (Amersham Pharmacia). The supernatant was added to the binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) as 1 ml HisTrap FF up to 50 ml. Thus the sample made was dripped into a vessel at 0.5 ml/min. After sample loading, the fusion was eluted by an eluting buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4), then desalted by Sephadex G-10 with desalting buffer (20 mM Tris, pH 7.8, 50 mM CH_3COONH_4) and lyophilized.

Antimicrobial assays

The purified msBD-1 and msBD-1-T peptides were dissolved in 20 mM Tris (pH 7.8, 200 μg/ml) and serially diluted at final concentrations of 25, 12.5, 6.3, and 3.1 μg/ml. The antimicrobial properties of recombinant msBD-1 were tested against *E. coli*, *S. aureus*, *P. vulgaris, P. aeruginosa*, and *S. flexneri.* The inhibitory zones of the two peptides were also measured.

Results

Construction of the msBD-1 and msBD-1-T cloning vectors

A 214-bp PCR product containing the sBD-1 sequence was cloned from the mucosa cells of sheep small intestine cDNA and inserted into the PMD19-T Simple vector to construct the cloning vector PMD19-T-sBD-1. Similarly, a 139-bp PCR product containing msBD-1 was obtained from PMD19-T-sBD-1, and inserted into the *Eco*RI and *Not*I sites of expression vector pPIC9 K to construct the pPIC9 K/msBD-1 vector. The plasmids were subsequently transformed in the *P. pastoris* GS115 expression host. HIS^+ transformants were selected by MM and MD plates, further identified by PCR using msBD-1-specific primers, and then confirmed by DNA sequencing. A schematic of pPIC9 K/msBD-1 and pPIC9 K/msBD-1-T expression plasmids are shown in Fig. 1.

Expression of the recombination protein from pPIC9 K/msBD-1 and pPIC9 K/msBD-1-T

Compared with the total proteins secreted by GS115 cells that were transformed with the empty pPIC9 K vector, an additional protein band with a molecular weight of approximately 3.8 kDa, which corresponded to the predicted size of msBD-1 and msBD-1-T products, was detected in the culture supernatants of all cells that were transformed with

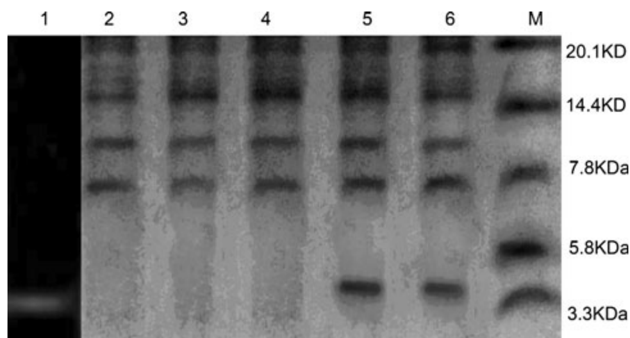

Fig. 2 Selection of msBD-1 and msBD-1-T producing colonies. Proteins were extracted from 20-μl samples of the induced culture supernatants and were either separated by Trince-SDS-PAGE and visualized by Coomassie Brilliant Blue staining (*lanes 2–6*) or analyzed by Western blotting on the msBD-1-T (*lane 1*) with the anti-His-HRP antibody. *Lane M* protein marker, *lane 2* a transformant containing the empty pPIC9 K vector; *lane 3* transformant containing the pPIC9 K/msBD-1 construct but uninduced by methanol, *lane 4* transformant containing the pPIC9 K/msBD-1-T construct but uninduced by methanol, *lane 5* transformant containing the pPIC9 K/msBD-1 construct and induced by methanol, *lane 6* transformant containing the pPIC9 K/msBD-1-T construct and induced by methanol

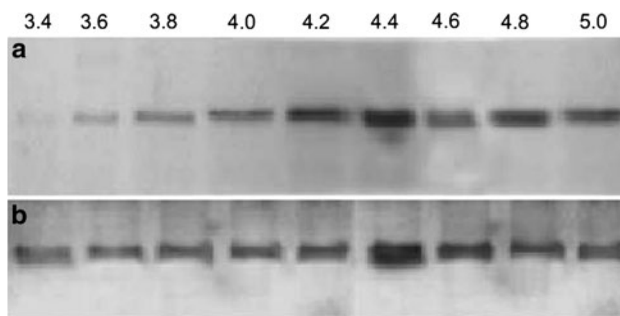

Fig. 3 Optimization of msBD-1 and msBD-1-T expression in pH (3.4, 3.6, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8, and 5.0). **a** msBD-1-T expression. **b** msBD-1 expression

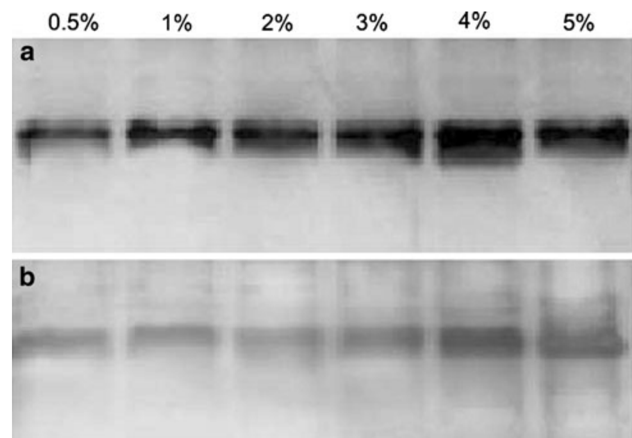

Fig. 4 Optimization of msBD-1 and msBD-1-T expression in methanol. **a** msBD-1-T expression. **b** msBD-1 expression

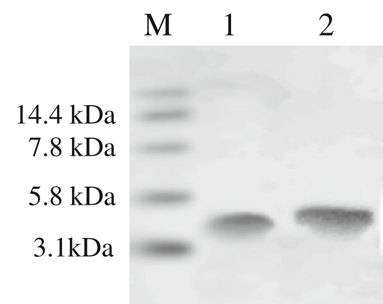

Fig. 5 The purification of msBD-1 and msBD-1-T. Two peptides were dissolved at 2.0 μg/ml, and to each lane was added 35 μl for Tricine-SDS-PAFE electrophoresis. The gel was stained by Coomassie Brilliant Blue. *Lane 1* msBD-1, *lane 2* msBD-1-T, *lane M* protein marker

msBD-1 and msBD-1-T genes. Western blotting performed using an anti-6-His tag antibody confirmed the msBD-1-T protein to be a His-tagged protein (Fig. 2). Therefore, we inferred that the msBD-1-T gene was successfully expressed by *P. pastoris.*

Because some proteins were specifically susceptible to the pH value, we examined whether the pH of the culture medium would affect the host cell growth of msBD-1 and msBD-1-T expression. Although the highest rate of cell growth following induction was observed at pH 7.0, the production of msBD-1 and msBD-1-T was most efficient at pH 4.4 (Fig. 3). Therefore, the culture was maintained at pH 4.4 as recommended in the manual provided with the *P. pastoris* expression kit.

The methanol concentration in the medium was an important factor that could affect the production of msBD-1 and msBD-1-T. The accumulation of msBD-1 and msBD-1-T was positively correlated with the density of viable cells at a methanol concentration of less than 3.0%. However, cell growth was inhibited under these conditions, and msBD-1 and msBD-1-T expression levels declined at a methanol concentration of 5.0%. In this experiment, however, the expression of both msBD-1 and msBD-1-T was effectively induced by the addition of methanol at a final concentration of 4.0% in the medium (Fig. 4).

Purification of msBD-1 and msBD-1-T

A total of 100 ml of yeast culture in BMMY medium (pH 4.4), yielding an initial OD_{600} value of 60, was induced by daily addition of methanol to a final concentration of 4.0%. After 2 days, soluble msBD-1 and msBD-1-T were secreted into the medium accounted for approximately 50 and 74% of the total extracellular protein, respectively. The msBD-1 protein was purified by SP-Sephadex (C-25) and the final msBD-1 yield was approximately 35 mg/l,

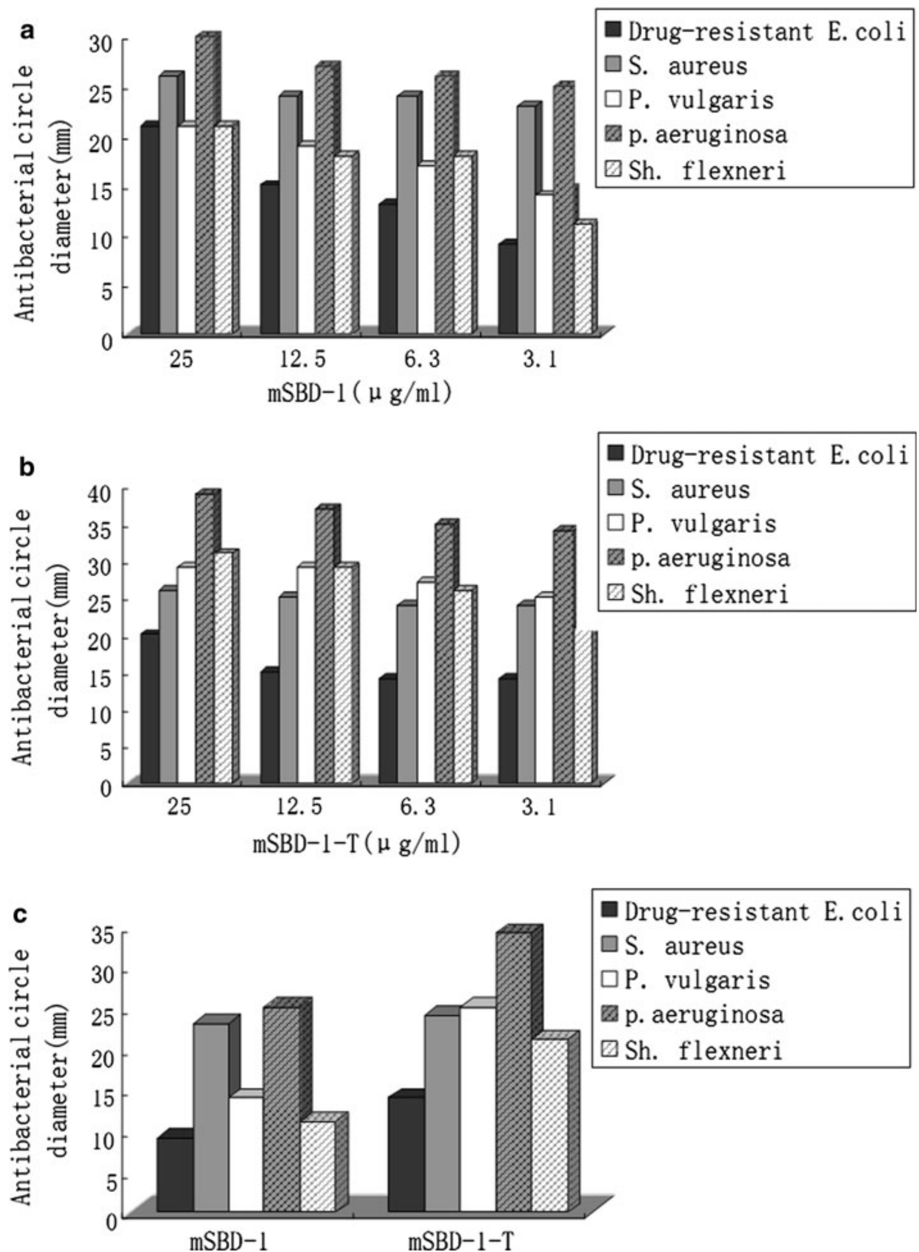

Fig. 6 Inhibition zones of msBD-1 and msBD-1-T on *E. coli*, *S. aureus*, *P. vulgaris*, *P. aeruginosa*, and *S. flexneri*: **a** msBD-1 and **b** msBD-1-T; **c** comparison of antimicrobial activity between msBD-1 and msBD-1-T at 3.2 μg/ml

resulting in a purification yield of 32%. On the other hand, due to the presence of the 6-His tag, msBD-1-T could be easily purified through Ni-agarose affinity chromatography followed by desalting using Sephadex G-10. The final msBD-1-T yield was approximately 80 mg/l with 85% purity (Fig. 5).

Antimicrobial assays

The antimicrobial properties of recombinant msBD-1 and msBD-1-T were tested against *E. coli*, *S. aureus*, *P. vulgaris, P. aeruginosa*, and *S. flexneri*. The inhibitory zones of the two peptides were also measured. In the inhibition zones, the concentrations of msBD-1 and msBD-1-T on *E. coli*, *S. aureus*, *P. vulgaris*, *P. aeruginosa*, and *S. flexneri* were 25, 12.5, 6.3, and 3.1 μg/ml, respectively (Fig. 6).

Discussion

When a substantial amount of sBD-1 was stored in Paneth cell granules in the form of a precursor peptide, very little msBD-1 can be successfully isolated from these cells, however, trypsin, which was also expressed in Paneth cells, can effectively cleave sBD-1 into msBD-1 in vitro [21]. Nonetheless, Ghosh et al. [10] have identified the predicted mature form of human nBD-5 in ileal luminal aspirates, supporting the notion that the cleaved product is indeed the

bioactive released form of this important peptide. Therefore, it is important to optimize methods for effective production of mature defensins, which was the aim of our work.

Previous studies have shown that many defensins are expressed in *E. coli* [15]. However, the fusion protein requires denaturation and refolding, which increases the difficulty of producing active defensins because the yield of bioactive defensins can greatly decrease through these complex processes. Furthermore, since msBD-1 is a relatively short peptide comprising only 38 amino acids, the addition of the 6-His tag could have affected its activity through steric hindrance. Therefore, we expressed msBD-1-T in *P. pastoris* and compared the antimicrobial activity of the purified product with the native non-6-His-tagged peptide in this study.

Because that pH value, methanol concentration, inoculum density of the culture medium, and time elapsed after induction can all affect the yield of proteins expressed in *P. pastoris*, the effects of these factors on cell growth and yield of the target peptides have also been investigated [12]. Although the magnitude of cell growth was not as large as that observed at lower pH values (e.g., pH 3.4–5.0), msBD-1 activity was often inhibited at this slightly acidic pH. This might explain why the accumulation of msBD-1 and msBD-1-T did not exhibit a correlation with cell growth when the pH of the culture medium was altered. In the case of the *P. pastoris* expression system, methanol is used for induction of protein expression and as the main carbon source for cell growth following induction. However, cell growth may also be inhibited by high concentrations of methanol [23]. In this study, a correlation between cell growth and yield of msBD-1 and msBD-1-T was observed when methanol was at a concentration of 0.5–5.0%, with the highest yield obtained at a concentration of 4.0%. During the first 48 h, msBD-1 and msBD-1-T production was high, but cell densities were reduced. However, after 48 h, the production of the peptides almost disappeared, while cell density increased. The exact reason for this observation is unclear at present.

To evaluate the antimicrobial activity of msBD-1 and msBD-1-T against pathogens and probiotic bacteria, *E. coli, S. aureus, P. vulgaris, P. aeruginosa*, and *S. flexneri* were used. Our results showed that the antimicrobial activity of msBD-1-T was higher than msBD-1. Interestingly, it has been shown that in avian β-defensin-8, increasing the positive amino acids at positively selected sites could enhance antimicrobial activity. Furthermore, in human β-defensin, Bohling et al. [1] showed that an α-helix was often presented in the C-terminus of β-defensins. In addition, although the C-terminal regions were more likely involved in membrane insertion and disruption of its aliphatic texture, only the region of the first eight amino acids in the C-terminal region showed antimicrobial activity [21]. Therefore, it was not surprising that our study revealed that msBD-1-T (which carries at the C-terminal the 6-His tag) also showed strong antimicrobial activity.

Conclusions

In summary, the presented study illustrated that both msBD-1 and msBD-1-T can be successfully expressed and purified from *P. Pastoris* while retaining their antimicrobial activity. The purification yield of msBD-1-T was higher than that of msBD-1, indicating that the addition of the 6-His tag to help the purification steps could be an effective strategy. Overall, the proposed approach may have a potential benefit for the further design of defensin-based therapeutic agents.

Acknowledgments On behalf of my co-authors, I submit the enclosed manuscript for consideration by the Journal. All authors have read and approved the submission of the manuscript to the "Journal of Industrial Microbiology and Biotechnology".

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