J Korean Soc Appl Biol Chem (2015) 58(6):807–812
DOI 10.1007/s13765-015-0109-8

Online ISSN 2234-344X
Print ISSN 1738-2203

NOTE

Recombinant expression of a novel antimicrobial peptide consisting of human α-defensin 5 and *Mytilus coruscus* mytilin-1 in *Escherichia coli*

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Received: 9 June 2015 / Accepted: 28 July 2015 / Published online: 6 August 2015

Abstract Antibiotic peptides are a battery of broad-spectrum antibacterial cationic polypeptides widely distributed in the plant and animal kingdoms. Among them, the human defensins are the first line of defense against pathogens and Mytilin, which is isolated from mussel serum, plays a key role in the mussel defense system. The antibacterial activity of these two peptides is generally ascribed to their overall positive charge, which enables them to disrupt bacterial membrane integrity and function. The aim of this study was to develop an effective method for the biosynthesis of a fusion peptide containing human α-defensin 5 (HD5) and Mytilin-1 in *Escherichia coli* to improve the antimicrobial activity. The individual HD5 and Mytilin-1 peptides were also synthesized for comparison with the fusion peptide. All the peptides, expressed as soluble fusions with the peptide thioredoxin, were isolated using a three-step purification strategy involving nickel-Sepharose chromatography, enterokinase cleavage, and cationic exchange chromatography. The identity of the peptides was confirmed by SDS-PAGE. Antimicrobial activity assays demonstrated that all the recombinant peptides had strong bactericidal properties and that the HD5 and Mytilin-1 fusion protein displayed higher activity against *E. coli* and *Staphylococcus aureus*. The results of this study provide a platform for the development of novel cationic peptides for biological studies.

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Keywords Expression · Human α-defensin 5 · Mytilin-1 · *Mytilus coruscus*

Introduction

Human α-defensin 5 (HD5) was initially found in Paneth cells in the small intestine and secreted into the gut lumen in response to bacterial stimulation (Jones and Bevins 1992). Subsequently, it was also detected in colon, genital mucosa, and other tissues (Frye et al. 2000; Spencer et al. 2012). HD5 was shown to possess strong bactericidal (Salzman et al. 2003; Ericksen et al. 2005) and antiviral activity both in vitro and in vivo (Buck et al. 2006; Hazrati et al. 2006; Furci et al. 2012; Wang et al. 2013; Hubert et al. 2014; Zins et al. 2014).

Mytilus coruscus, the hard-shelled mussel, belongs to the family *Mytilidae*. It is one of the most important marine shellfish species and is widely cultured throughout coastal areas of China. Mytilin which is an important antibacterial peptide isolated from the mussel serum has been demonstrated to have broad-spectrum activity against Gram-positive and Gram-negative bacteria (Charlet et al. 1996). Structural and functional investigations of Mytilin indicated that the fragment connecting two β-sheets in a stable β-hairpin structure is required for antimicrobial activity. The cDNA gene sequence of Mytilin-1 has been determined (GenBank accession number: FJ973154) and was used in the current study as a representative of the Mytilins from *M. coruscus*.

Based on the strong bactericidal activity of HD5 and the antimicrobial activity of Mytilin, we hypothesized that the two peptides in combination will exert a synergistic effect. Due to practical difficulties and the high cost of obtaining

Table 1 Sequence of primers P1–P4 used to amplify the mHD5, mMy1, and mHM genes codon optimized for expression in *E. coli*

Oligo no.	Oligo sequence (from 5′ end to 3′ end)
P1	GGTACCGACGACGACGACAAGGCCACCTGCTACTGCCGC
P2	GGATCCTTATTAACGGCAGCACAGACGGTACAGGCG
P3	GGTACCGACGACGACGACAAGAGCTGCGCCAGCGTTTGC
P4	GGATCCTTATTAGCAACGCAGGCAGCGGCAATAGCAG

mature HD5 (mHD5) and mature Mytilin-1 (mMy1) from natural sources or through chemical synthesis, genetic engineering represents a promising solution to the cost-effective production of large amounts of peptides. The prokaryotic expression system is by far the simplest and most inexpensive means of producing heterogeneous proteins. The *Escherichia coli* (*E. coli*) expression system has been used successfully to produce a number of bioactive antimicrobial peptides such as human defensin (Wang et al. 2010; Chapnik et al. 2012; Coordes et al. 2012; Seo et al. 2012). In the present study, we first constructed recombinant plasmids for expression of the individual peptides (pET32a-mHD5 and pET32a-mMy1) and the fusion peptide, pET32a-mHD5-mMy1 (pET32a-mHM). The corresponding genes were codon optimized for expression in *E. coli* and preceded by an enterokinase recognition sequence and then inserted into the expression vector pET32a, in frame with the upstream thioredoxin (TrxA) gene and His-tag. The conditions for soluble expression of these fusion proteins at high levels were optimized in preliminary experiments before the purification strategy was developed to obtain the three recombinant proteins. Each peptide was identified by SDS-PAGE and their biological activity was evaluated in bactericidal assays.

Materials and methods

Strains, plasmids, and enzymes

Escherichia coli DH5α (TaKaRa, China) was used as the gene manipulation host. BL21(DE3) competent *E. coli* cells (Tiangen, China) were used as the host for expression of the heterologous protein. The plasmid of pET32a (Novagen, Germany) was used to construct the expression vector. DNA polymerase, T4 DNA ligase, *Kpn*I, and *Bam*HI were purchased from TaKaRa.

Design of the mHD5, mMy1, and mHD5-mMy1 (mHM) fusion genes codon optimized for expression in *E. coli*

The sequences of mHD5 (GenBank accession no. M97 925) and mMy1 (GenBank accession no. FJ973154) were optimized according to the *E. coli* codon usage table (http://www.kazusa.or.jp/codon/). These two sequences were fused by a sequence encoding a poly-Gly linker to allow flexibility of the peptide. The codon-optimized mHD5, mMy1, and mHM genes were produced by Genewiz (China). All primers designed for and used in this study are listed in Table 1.

Construction of the recombinant plasmids

Primers P1 (harboring a *Kpn*I site) and P2 (harboring a *Bam*HI site) were used for amplification of the mHD5 gene fragment using the synthesized mHD5 gene as a template and subsequent ligation into *Kpn*I/*Bam*HI-cut pET32a(+). Polymerase chain reaction was performed as follows: preheating at 94 °C for 5 min, 25 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s, followed by an elongation at 72 °C for 10 min. The recombinant plasmid pET32a-mHD5 encodes the fusion protein consisting of mature HD5 with the upstream TrxA gene and a His-tag, which facilitates the purification and identification of the fusion proteins. A similar procedure was used for the amplification and cloning of the mMy1 gene with primers P3 and P4 to generate pET32a-mMy1. This plasmid encodes mature Mytilin-1 with the upstream TrxA gene and a His-tag. In the same way, plasmid pET32a-mHM encoding the proform of mature HD5-Mytilin1, with the upstream TrxA gene and a His-tag, was constructed using primers P1 and P4 (Table 1). Primers P1 and P3 were designed to incorporate the enterokinase cleavage sequence at the N-terminal to facilitate production of the target protein.

For construction of the recombinant plasmids, the amplified DNA fragments generated were isolated by agarose gel electrophoresis. The eluted PCR products and pET32a(+) were then digested with *Kpn*I and *Bam*HI and purified for ligation using T4 ligase to generate pET32a-mHD5, pET32a-mMy1, and pET32a-mHM.

Expression and purification of fusion proteins

To optimize the conditions for expression of the recombinant proteins, diverse factors were analyzed, including cell density at induction (OD_{600} = 0.1–1.0), IPTG concentration (0.1–1.0 mM), incubation time (1–12 h), and temperature (20–37 °C). BL21(DE3) cells were transformed with

recombinant pET32a-mHD5, pET32a-mMy1, and pET32a-mHM. Each fresh clone of the host strain harboring the expression vector was initially cultured in Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin to an optical density (OD_{600}) of 0.5. The culture was then transferred into 25 mL LB medium containing 1 % glucose per 250-mL flask at a ratio of 10 % (v/v) for culture at 37 °C, 250 rpm, until the OD_{600} reached 1.0. Subsequently, the induction was initiated with 0.5 mM IPTG at 30 °C for 4 h. The cells were harvested by centrifugation at 5000×*g* for 10 min and then suspended in 20 mM Tris-HCl (pH 7.5) prior to lysis by sonication in an ice-water bath. The soluble and insoluble proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

After centrifugation at 12,000×*g* for 15 min, the supernatant of the cell lysate was directly applied to a nickel-Sepharose high-performance column (XK16/20, GE Healthcare) that had been equilibrated with binding buffer (20 mM Na_3PO_4, 500 mM NaCl, 5 mM imidazole, pH 7.4). The proteins were then eluted with a linear gradient of imidazole. The eluted fractions were analyzed by SDS-PAGE. The Bradford assay was used for quantitative analysis of the proteins.

Release and purification of target proteins

The target protein was released from the fusion protein by digestion with enterokinase according to the manufacturer's instruction. The digested reaction mixture was applied to a SP ion-exchange chromatography column pre-equilibrated with five column volumes of phosphate-buffered saline (PBS, pH 7.0) to wash out the unwanted fractions. The target protein was then eluted from the column with a linear gradient of 0–1.0 NaCl PBS. The purified target proteins were identified by SDS-PAGE.

Antimicrobial activity assays

The antimicrobial activity of the purified recombinant mHD5, mMy1, and mHM was tested against *E. coli* strain ATCC 25922 and *S. aureus* ATCC 29213 using assays that were essentially the same as those described previously for defensins (Harwig et al. 1994). The bacteria were cultured at 37 °C in tryptic soy broth to mid-log phase and then diluted to 1×10^6 cfu/mL in 10 mM potassium phosphate, 1 % tryptic soy broth (pH 7.4). The bacterial cultures were then incubated in the presence of different concentrations of the peptides for 3 h at 37 °C. The cultures were then serially diluted using the same buffer and plated on LB agar plates. After incubation for 18 h at 30 °C, individual colonies of bacteria were counted. Bacterial activity was expressed as the ratio of colonies counted to the number of colonies on a control plate. LD_{50}, LD_{90}, and LD_{99} are defined as the minimum tested concentration of peptide at which at least 50, 90, and 99 % of the viable cells are killed, respectively.

Statistical analysis

Data are presented as the means ± the standard deviation (SD) of three or more independent experiments. The statistical significance was assessed by student's *t* test, with $p < 0.05$ considered significant.

Results

Codon optimization and construction of expression vectors

Differences in codon usage between species can affect the quantity and quality of the proteins expressed by recombinant techniques. Therefore, codons in the cDNA of mature HD5 and My1 were analyzed for rarely occurring codons.

mHD5 (GenBank accession no. M97925) consists of 32 amino acids, 19 amino acids (59.4 %) of which are encoded by rarely used codons in *E. coli* (Fig. 1A). mMy1 (GenBank accession no. FJ973154) consists of 34 amino acids with 23 amino acids (67.6 %) encoded by low-usage codons in *E. coli* (Fig. 1B). To avoid the potential problems of these rare codons in the expression of mHD5 and mMy1 in *E. coli*, the rarely used codons were replaced by frequently used

Fig. 1 Comparison of (**A**) mHD5 and (**B**) mMy1 sequences between original cDNA and optimized codons. *Lane 1* optimized sequences of (**A**) mHD5 gene, and (**B**) mMy1 gene; *Lane 2* original cDNA sequences of (**A**) mHD5 gene, and (**B**) mMy1 gene

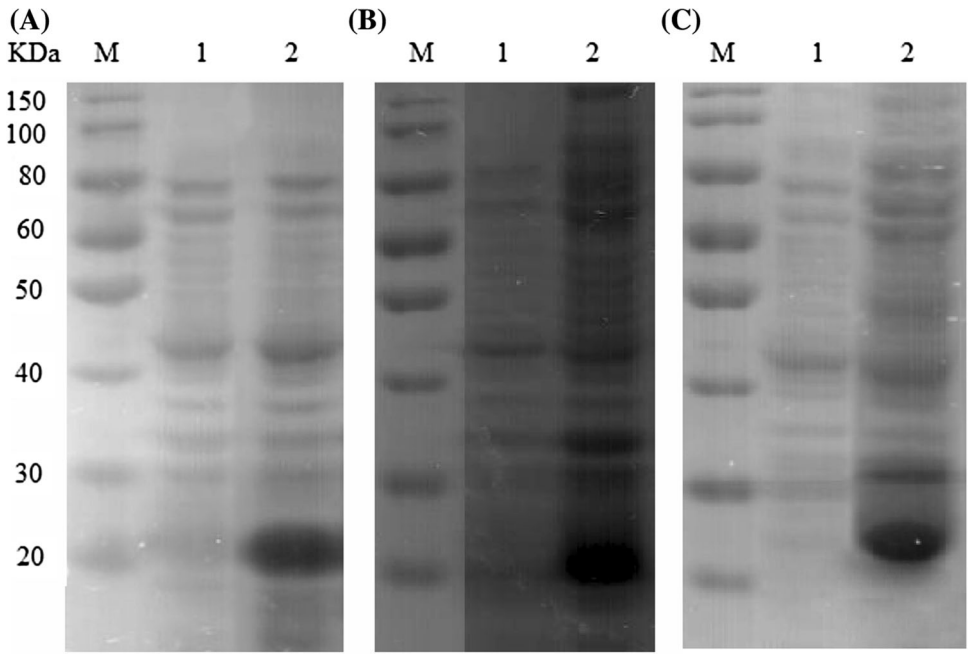

Fig. 2 SDS-PAGE analysis of (**A**) recombinant mHD5, (**B**) mMy1, and (**C**) mHM expressed in *E. coli* BL21. *Lane M*, protein molecular weight marker; *Lane 1* bacterial lysate containing recombinant expression vector before IPTG induction; *Lane 2* bacterial lysate containing recombinant expression vector after IPTG induction for 4 h

ones, according to the codon usage table of *E. coli*. Additionally, a tandem repeat of TAA was added at the C-terminal because this is the most efficient translation termination sequence in *E. coli*. The codon-optimized mHD5 and mMy1 genes were fused by a sequence encoding a poly-Gly linker.

Based on the codon-optimized sequences, four oligonucleotide primers (Table 1) were designed and used to clone mHD5, mMy1, and mHM. These DNA fragments were obtained by PCR amplification and were cloned into the pET32a fusion expression vector after a sequence encoding an enterokinase cleavage site with the 6× His-tag sequence at the carboxyl terminus of the thioredoxin gene.

Expression and purification of fusion protein

Bacterial cells harboring the recombinant plasmid were analyzed for expression of the fusion protein by 10 % SDS-PAGE (Fig. 2). The mHD5 and mMy1 proteins were observed as the fused status (MW 22 kDa) on the c-terminal of Trx protein, respectively. And mHM protein was detected as a fusion protein (Trx-mHD5-mMy1; MW 26 kDa). These apparent molecular weights were consistent with their theoretical molecular weights. All of the recombinant proteins were detected in the supernatant as well as in the pellet. Utilizing the characteristics of the His-tag and the isoelectric point of the fusion protein, an efficient three-step purification strategy comprising affinity chromatography, enzyme digestion, and ion-exchange chromatography was successfully developed to purify the recombinant proteins. Following IPTG induction, the bacterial cells transfected with

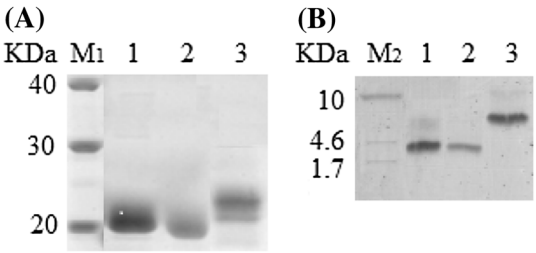

Fig. 3 SDS-PAGE analysis of (**A**) the purified fusion proteins and (**B**) target peptides. (**A**) *Lane* M_1 protein molecular weight marker; *Lanes 1, 2*, and *3* TrxA-mHD5, TrxA-mMy1, and TrxA-mHM fusion proteins, respectively, purified by nickel-Sepharose chromatography. (**B**) *Lane* M_2 protein molecular weight marker; *Lanes 1, 2*, and *3* mHD5, mMy1, and mHM target peptides, respectively

pET32a-mHD5, pET32a-mMy1, and pET32a-mHM were lysed and the supernatants were subjected to nickel-Sepharose chromatography. The proteins contained within the fraction eluted with 0.3 mM imidazole were analyzed by SDS-PAGE (Fig. 3A). The location of these three recombinant proteins was consistent with the results of preceding analysis (Fig. 2), indicating that the purification of these target proteins was successful. The yield of these fusion proteins exceeded 1.45 g/L, with a purity of 84.5 % in all cases. After cleavage with enterokinase, the released mHD5 (4 kDa), mMy1 (4 kDa), and mHM (8 kDa) peptides were effectively separated from other components by ion-exchange chromatography and identified by SDS-PAGE (Fig. 3B). Finally, the purified recombinant peptides were prepared for use by desalting and freeze drying, with yields of approximately 90 mg/L.

Table 2 Antibacterial activity of recombinant mHD5, mMy1, and mHM against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 as determined by colony counting

	Strain	LD_{50} (μg/mL)	LD_{90} (μg/mL)	LD_{99} (μg/mL)
mHD5	*E.coli*	3.1 ± 1.4*	7.9 ± 2.7*	22.1 ± 4.5*
	S.aureus	3.9 ± 1.3*	31.6 ± 6.9*	76.0 ± 0.0*
mMy1	*E.coli*	8.9 ± 3.7*	15.8 ± 5.4*	44.3 ± 4.0*
	S.aureus	2.7 ± 1.7	6.9 ± 2.3	34.2 ± 6.5
mHM	*E.coli*	1.1 ± 0.3	3.1 ± 1.3	9.3 ± 2.7
	S.aureus	0.9 ± 0.2	3.7 ± 1.2	12.6 ± 6.6

* Significant difference ($p < 0.05$) compared with mHM

Antimicrobial activity assay

The antimicrobial activity of recombinant mHD5, mMy1, and mHM was tested against two different bacterial strains, *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 using colony counting methods. As shown in Table 2, the recombinant peptides were characterized by strong bactericidal properties. Recombinant mHM exerted significantly higher antibacterial activity against both strains compared with mHD5. The mHM peptide also exerted significantly higher antibacterial activity against *E. coli* compared with mMy1. However, although the anti-*S. aureus* activity of mHM was greater than that of mMy1, the difference was not significant.

Discussion

Escherichia coli is the most commonly used host for the expression of heterologous proteins, because of its fast growth rate and well-established expression system. However, there are two major barriers to the expression of small cationic peptides with antibacterial activity in bacteria: the potential ability of the cationic peptides to kill the host strain and the susceptibility of the cationic peptides to proteolytic degradation. The use of a fusion protein expression system has been found to overcome such barriers (Piers et al. 1993). Adopting this approach in this study, the target gene was ligated into the pET32a(+) expression vector in a way that gives rise to a fusion gene encoding a TrxA protein, which has been shown to be suitable for cationic peptide expression (Rao et al. 2004). Using this system, we obtained high expression levels of TrxA-mHD5, TrxA-mHy1, and TrxA-mHM; and more importantly, these target proteins were expressed in a soluble form in *E. coli* cells. TrxA has also been reported to be beneficial in the correct folding and solubility of heterogeneous proteins expressed in *E. coli* (de Marco 2009), which may contribute to the bioactive potential of the recombinant proteins.

In this study, mHD5, mMy1, and mHM were expressed at relatively high levels using this system. Mytilin-1 derived from *M. coruscus* was first cloned and produced in *E.coli*. *M. coruscus* belongs to the family Mytilidae, a major species of cultured marine shellfish, which has been used as food and medicine for thousands of years. The research on antibacterial peptides from *M. coruscus* has been of significant value in the further exploitation of antibacterial peptides.

Analysis of the antimicrobial activity (Table 2) showed the purified recombinant peptides provided strong, dose-dependent antimicrobial effects against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. The antibacterial activity of recombinant mHD5 against *E. coli* and *S. aureus* was similar to the published data determined for oligonucleotide (Pazgier and Lubkowski 2006) and solid-phase peptide synthesized (Wu et al. 2004) defensin HD5. The recombinant mMy1 also exhibited antibacterial activity consistent with that of the synthetic peptide (Roch et al. 2008). It is especially worth noting that the recombinant mHM displayed greater antibacterial activity compared with the recombinant peptides expressed as individual components; thus, demonstrating that these two antibiotic peptides expressed as a fusion protein exert a synergistic effect. The mature forms of both HD5 and Mytilin are arginine-rich proteins (Brogden et al. 1996); therefore, recombinant mHM is comparatively richer in arginine residues and carries an even greater positive charge. It can be speculated that this characteristic of the recombinant mHM fusion protein enhances its capacity to bind to and damage the negatively charged bacterial membrane (McPhee and Hancock 2005). However, the mechanism by which the peptide mediates antibacterial activity remains to be fully elucidated.

In summary, we have successfully achieved the expression and purification of soluble mHD5, mMy1, and mHM fusion peptides in *E. coli*. Moreover, this is the first report of the efficient preparation of bioactive Mytilin-1 from *M. coruscus* and the expression of a soluble fusion peptide comprising mHD5 and mMy1 in *E. coli*. The strategies established in this study provide a platform for further structural and functional studies of these antibiotic peptides.

Acknowledgments This work was supported by the Natural Science Foundation of Liaoning Province of China [Grant Number 201202140].

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