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Simple Purification of the Human Antimicrobial Peptide Dermcidin (MDCD-1L) by Intein-Mediated Expression in *E. coli*

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Among human antimicrobial peptides (hAMPs), DCD-1L has a broad spectrum of antimicrobial activity over a wide pH range and in high salt concentrations. It offers a promising alternative to conventional antibiotics. The 458-bp-long dermcidin cDNA was amplified by PCR using a human fetal cDNA library as a template. The 147-bp fragment of the MDCD-1L gene encoding an additional methionine residue was subcloned into the pTYB11 vector. Recombinant MDCD-1L was expressed as an intein fusion protein in *E. coli*, and then purified by affinity chromatography using chitin beads. A small peptide with a molecular mass of about 5 kDa was detected by tricine gel electrophoresis. The recombinant MDCD-1L peptide was purified from the gel and its amino acid sequence was determined by nanoLC–ESI–MS/MS analysis. The initiating amino acid, methionine, remained attached to the N-terminal region of recombinant MDCD-1L. Purified MDCD-1L showed antimicrobial activity against a *Micrococcus luteus* test strain.

Keywords: Antimicrobial peptides, DCD-1L, dermcidin, intein, protein expression

Dermcidin (DCD) is a broad-spectrum antimicrobial peptide (AMP) that is produced by eccrine glands and secreted in sweat [13, 15]. After its expression, the 110-amino-acid-long dermcidin is proteolytically processed to generate the peptides DCD-1, composed of 47 amino acids, and DCD-1L, containing 48 residues, as well as shorter fragments. DCD-1L exhibits a broad spectrum of antimicrobial activity over a wide pH range and in high salt concentrations [15]. In contrast to most other AMPs, which are cationic, DCD has a net negative charge. Interestingly, proteolytically processed DCD peptides possess net charges between −2 and +2. Neither the cationic nor anionic DCD peptides permeabilize the inner and outer membranes of *E. coli* [19]. The mode of action of DCD-derived peptides is different from that of the cathelicidin LL-37. According to Steffen *et al.* [19], the antimicrobial activity of DCD peptides is not due to pore formation or destabilization of the bacterial membrane [1, 6, 10, 18]. Instead, anionic and cationic DCD-derived peptides exhibit a time-dependent bactericidal activity that is followed by bacterial membrane depolarization [17]. These characteristics make DCD peptides suitable for use as viable alternatives to conventional synthetic antibiotics [2, 12, 16].

Previously, recombinant DCD peptides (DCD-1 and DCD-1L) were purified as fusion proteins using thioredoxin or ketosteroid isomerase as fusion partners. Recombinant DCD-1L has been expressed in *E. coli* as a fusion protein and subsequently purified [10]. Čipáková *et al.* [4] reported DCD-1 expression with a C-terminal homoserine lactone (DCD-1Hsl) and 6×His in *E. coli*. This fusion protein was expressed as an insoluble protein, which was made soluble by CNBr-mediated cleavage and then purified. However, all of these fusion expression studies required a tedious cleavage step and an additional purification step.

Recently, intein-mediated expression of some AMPs was successfully employed [3, 5, 11]. Intein-mediated expression/purification of proteins is based on the fusion of the target protein to an intein tag that is self-cleavable by a simple pH change or thiol-group addition. Inteins are considered to be the protein analogs of introns, owing to their ability to induce their own excision from a precursor polypeptide as well as the in-frame fusion of flanking protein sequences (*viz.*, exteins) to give a functionally mature protein. The main advantage of the intein protein purification strategy resides in the fact that recombinant proteins may be recovered free of the affinity tag through a single-step cleavage reaction. Morassutti *et al.* [11] reported the

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production of the cathelicidin-like peptide SMAP-29, using an intein fusion expression system.

The aim of the present study was to demonstrate that an intein can be used successfully as a fusion partner for purification of the DCD-1L antimicrobial peptide by single-step intein-mediated splicing. Based on this mechanism, the purification of DCD-1L can be achieved using a chitin-binding domain (CBD) affinity tag and thioester bond cleavage with 1,4-dithiothreitol (DTT). Herein, we attempted to produce DCD-1L using an intein-mediated fusion expression system. The purified peptide was analyzed by mass spectrometry and its antimicrobial activity was confirmed using a radial diffusion assay.

MATERIALS AND METHODS

Strains, Culture Media, and Recombinant DNA Techniques

E. coli bacterial strain XL10-Gold (Stratagene, U.S.A.) was used as the host for plasmid constructions. *E. coli* BL21 (DE3) (Novagen, Germany) and ER2556 (New England Biolabs, U.K.) were used as hosts for protein expression. These strains were cultured in Luria–Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% sodium chloride) supplemented with ampicillin (50 μg/ml) or zeocin (25 μg/ml). For solid media, 1.5% agar was used. All media components were manufactured by Difco Co. (U.S.A.), and all enzymes were purchased from Takara (Japan) unless otherwise noted. The *E. coli* expression vector pTYB11 was purchased from New England Biolabs. Restriction enzyme digestions, plasmid engineering, and standard techniques were performed as specified elsewhere [14]. Plasmid DNA was prepared for sequencing using a DNA-spin plasmid DNA purification kit (iNtRON Biotechnology Co., Korea), DNA fragments were recovered from agarose gels using a MEGA-spin agarose gel extraction kit (iNtRON), and PCR products were purified using a PCRquick-spin PCR product purification kit (iNtRON), all as described in the manufacturer's manuals. *E. coli* transformation was performed by the $CaCl_2$ method. *Micrococcus luteus* IAM1053 (KCCM11576T) was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea) for use as a test strain for antimicrobial activity and was maintained in nutrient broth (NB) containing 20% (v/v) glycerol at −70°C.

PCR Amplification and Cloning of Human Dermcidin Gene

PCR amplification of AMP genes was performed in a Geneamp PCR system 2700 (Applied Biostems, U.S.A.) and a Gene Cycler (Bio-Rad, U.S.A.). The 513-bp-long human cathelicidin cDNA coding region (GenBank Accession No. NM004345) was synthesized by touchdown PCR using a human fetal cDNA library as a template and 40 cycles of 10 s at 98°C, 1 min at 62°C /50°C, and 1 min at 72°C following an initial denaturation for 5 min at 94°C. The dermcidin gene was amplified using PCR primers DCDfull-F (5'-CCAAGATCTCCAAGGATT-3') and DCDfull-R (5'-AGGTTTTAG GCTGAAGACGTAAAGC-3'). PCR products were ligated into the pGEM-T Easy Vector (Promega, U.S.A.), the sequence of which was verified by DNA sequencing. The 147-bp DCD-1L gene was amplified by nested PCR using the internal primers MDCD-1L (*Sap*I)-F (5'-GGTGGTTGCTCTTCCAACATGTCCAG CCTTCTGG AAA-3') and DCD-1L (*Not*I)-R (5'-GCGGCCGCCTATAGTACTGA GT CAAG-3'). An initial denaturation was carried out at 94°C for 1 min, followed by 28 cycles consisting of denaturation at 94°C for 15 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and an additional final extension at 72°C for 7 min. The purified PCR product and pTYB11 vector DNA were digested with *Sap*I and *Not*I and then ligated. The ligation product was transformed into XL10-gold.

Purification of Recombinant Peptide MDCD-1L

All purification steps were performed at 4°C. To purify the intein–MDCD-1L fusion protein, *E. coli* ER2556 harboring pTYB11/MDCD-1L was grown in 1 l of LB medium containing 50 μg/ml ampicillin to an absorbance of approximately 0.6 at 600 nm. The culture was then adjusted to 0.4 mM IPTG, and incubation was continued at 30°C for 9 h. The cells were harvested by centrifugation at 10,000 rpm for 10 min, resuspended in 40 ml of column buffer (20 mM sodium phosphate buffer, 0.5 M NaCl, pH 7.5), washed twice, and then resuspended in 25 ml of the same buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell suspension was placed on ice and sonicated using an Artec Sys. Co-150 ultrasonicator. The sonicated cells were then centrifuged at 15,000 rpm for 10 min, and protein was purified from the supernatant using a Bio-Rad Econo-Column (2.5×10 cm). Twenty ml of chitin beads (New England Biolabs, Inc.) was packed in the column, and then washed with two volumes of column buffer using gravity flow. Forty ml of sonicated protein solution was loaded and passed through the column. After this binding process, the chitin beads were washed with 10 volumes of column buffer. For induction of cleavage between the CBD and the MDCD-1L peptide, two volumes of elution buffer (20 mM sodium phosphate buffer, 0.5 M NaCl, and 100 mM β-mercaptoethanol, pH 7.5) were passed through the column, and then the column flow was stopped. The MDCD-1L peptide was cleaved for 16 h at 4°C. The elution process was carried out by the addition of 10 ml of column buffer. The eluted peptide was concentrated by the addition of two volumes of ethanol, and then dialyzed overnight against distilled water using benzoylated dialysis tubing (Sigma) to remove the buffer components and β-mercaptoethanol. The purified peptides were separated on a tricine gel.

NanoLC–ESI–MS/MS Analysis for Protein Identification

An approximately 5-kDa band detected on SDS–PAGE was manually excised from the gel. The gel pieces were subsequently treated with destaining solution (100 mM sodium thiosulfate, 30 mM potassium ferricyanate), washed with milli-Q water, and rehydrated with 200 mM ammonium bicarbonate. The pieces were then washed several times with milli-Q water for 15 min each, dehydrated with acetonitrile for 5 min, dried, and rehydrated with a total of 200–300 ng of sequencing-grade modified trypsin (Promega, Madison, WI, U.S.A.) in 25 mM ammonium bicarbonate, pH 8.5, at 37°C for 16 h. Following digestion, tryptic peptides were extracted twice with 50% (v/v) acetonitrile containing 0.5% (v/v) trifluoroacetic acid (TFA) for 15 min with moderate vortexing. The extracts were pooled and evaporated to dryness under vacuum. For ESI–MS/MS analysis, dried peptide samples were redissolved in 0.1% (v/v) TFA and purified by C18 Zip-Tip chromatography (Millipore, Billerica, MA, U.S.A.) according to the manufacturer's instructions.

Direct 1D nanoLC–ESI–MS/MS analysis was performed on an integrated nanoLC–MS/MS system (Waters Co., Japan) comprising

a three-pump Micromass/Waters CapLC system with an autosampler, a stream select module configured for a precolumn plus an analytical capillary column, and a Micromass Q-Tof Global mass spectrometer fitted with a nano-LC sprayer, operated under the control of MassLynx 4.0 (Micromass, Manchester, U.K.).

Antimicrobial Analysis of Recombinant Protein

Antimicrobial activity was measured by a radial diffusion assay. Autoclaved NB containing 1.5% agar was cooled to 45°C and poured into 9-cm-diameter. Petri dishes containing a 0.1% dilution of an overnight culture of the test strain, *M. luteus* IAM1056. After the agar solidified, sterile cylindrical tubes (6 mm inner diameter×10 mm height) filled with 200 μl of recombinant protein samples were placed against the bacterial lawn on the plates, and then incubated at 30°C for 1 day. Antimicrobial activity was assessed by determining the size of clear zones that formed around the tubes.

Results

E. coli Expression Vector Construction

The target DCD-1L AMP gene was amplified and ligated into pTYB11 by digestion with *Sap*I and *Not*I. Because of the efficiency of cleavage between the intein and the AMP, the forward primer DCD-1L (*Sap*I)-F encodes an extra methionine residue at the N-terminus of the AMP. Successful construction of the expression vector, pTYB11/MDCD-1L, was confirmed by colony PCR and enzyme cleavage. The resultant recombinant plasmid used for expression of MDCD-1L is shown in Fig. 1.

Fusion Protein Expression in *E. coli* ER2556 and Purification of MDCD-1L from the Intein Fusion Protein

The plasmid pTYB11/MDCD-1L was transformed into *E.coli* ER2556, and pTYB11/MDCD-1L-positive clones were used to overexpress the intein–MDCD-1L fusion protein. After 12 h of induction with 0.3 mM IPTG at 30°C, recombinant proteins were analyzed by SDS–PAGE (Fig. 2). An approximately 61-kDa intein–MDCD-1L fusion protein was successfully expressed in soluble form. As part of the intein fusion protein, MDCD-1L had no detectable cytotoxic activity toward host cells, so the expression of fusion protein did not influence bacterial growth. The results demonstrate that IPTG induced the expression of high levels of soluble intein–MDCD-1L

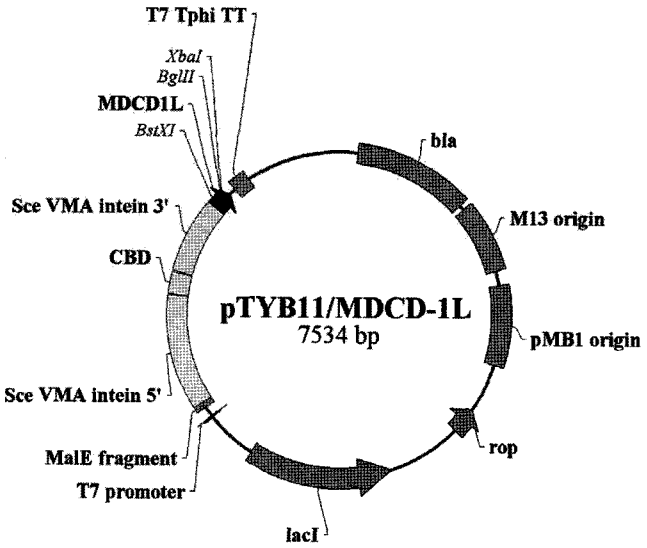


Fig. 1. Map of the pTYB11 vector with the intein–MDCD-1L insert.

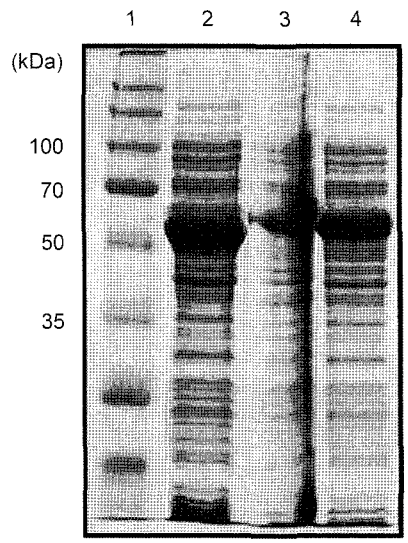


Fig. 2. SDS–PAGE analysis of fusion protein expression.
Lane 1, molecular size marker; lane 2, induced pTYB11 vector, soluble fraction; lane 3, induced pTYB11/MDCD-1L, total lysate; lane 4, induced pTYB11/MDCD-1L, soluble fraction.

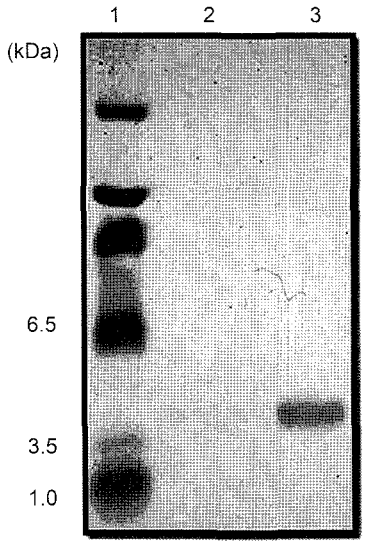


Fig. 3. Tricine gel electrophoresis of purified MDCD-1L.
Lane 1, low range molecular mass marker; lane 2, pTYB11 vector control; lane 3, pTYB11/MDCD-1L.

fusion protein without affecting the viability of bacterial cultures. The MDCD-1L peptide was purified to homogeneity in a single step using chitin affinity chromatography. After cell disruption, the intein–MDCD-1L fusion protein was bound to chitin beads, and then extensive washing was performed to remove nonspecific proteins. After the incubation of the cleavage reaction with β-mercaptoethanol, the MDCD-1L was released from the intein-CBD tag and was eluted from the column in the column buffer since the CBD could not be eluted from the chitin resin under non-denaturing conditions. The β-mercaptoethanol was removed by dialysis. The target peptide was detected by tricine gel electrophoresis (Fig. 3). Ten mg of purified MDCD-1L peptide was lyophilized and stored at 4°C.

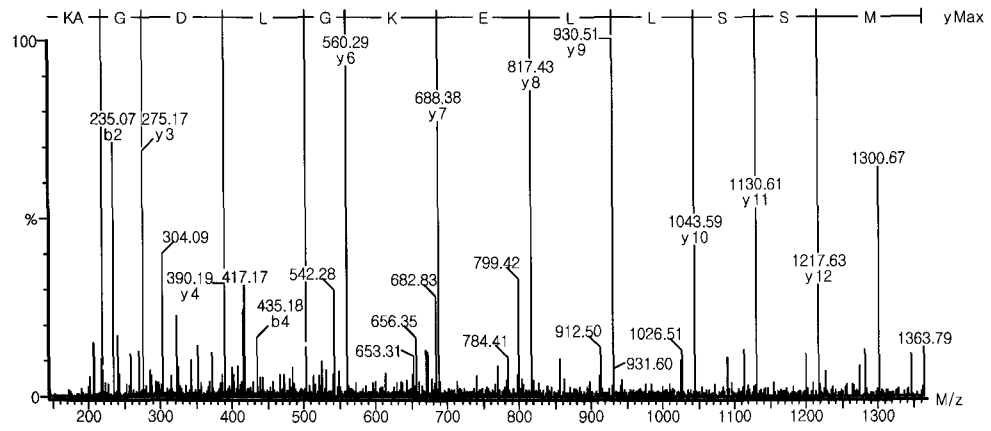
NanoLC–ESI–MS/MS Analysis for Protein Identification

In order to determine whether the recombinant MDCD-1L gene product was the same as authentic MDCD-1L, the approximately 5-kDa peptide detected on SDS–PAGE was digested with trypsin, and the resulting fragments were characterized by nanoLC–ESI–MS/MS analysis. Mass data measured for the five peptide fragments were 412.191 (Δ mass=0.0381), 682.835 (Δ mass=0.0361), 498.259 (Δ mass=0.0241), 601.335 (Δ mass=0.0244), and 730.363 (Δ mass=0.0305), which correspond to the expected masses for the peptides MSSLLEK, MSSLLEKGLDGAK, MSSLLEKGLDGAKK, AVGGLGK, and LGKDAVEDLESVGK, respectively. Therefore, tryptic peptide analysis indicates that the recombinant MDCD-1L peptide was authentic DCD-1L with an attached N-terminal methionine (Fig. 4).

Bioassay of Recombinant DCD-1L

To evaluate the antimicrobial activity of MDCD-1L, cylindrical tubes filled with purified recombinant peptide were tested against a bacterial lawn of *M. luteus* IAM5350 as described above. A clear zone appeared around tubes containing recombinant MDCD-1L whereas none formed around the negative control (Fig. 5).

A



B

site	Precursor m/Z	Delta mass	Peptide sequence
1-7	412.191	0.0381	MSSLLEK
1-13	682.835	0.0361	MSSLLEKGLDGAK
1-14	498.259	0.0241	MSSLLEKGLDGAKK
15-21	601.335	0.0244	AVGGLGK
22-35	730.363	0.0305	LGKDAVEDLESVGK

C

DCD-1L SSLLEKGLDGAKKAVGGLGKLGKDAVEDLESVGKGAVHDVKDVLDSVL

rDCD-1L MSSLLEKGLDGAKKAVGGLGKLGKDAVEDLESVGKGAVHDVKDVLDSVL

Fig. 4. NanoLC-ESI MS/MS analysis of recombinant MDCD-1L.

Mass analyses of several peptides indicate that recombinant MDCD-1L peptide was authentic DCD-1L with an N-terminal methionine attached. Panel (**A**) MS/MS data of MDCD-1L fragments generated from peptide (1)MSSLLEKGLDGAK(13); Panel (**B**) mass data of several peptides from trypsin digestion; Panel (**C**) peptide sequence of recombinant MDCD-1L. Identified peptide sequences were underlined.

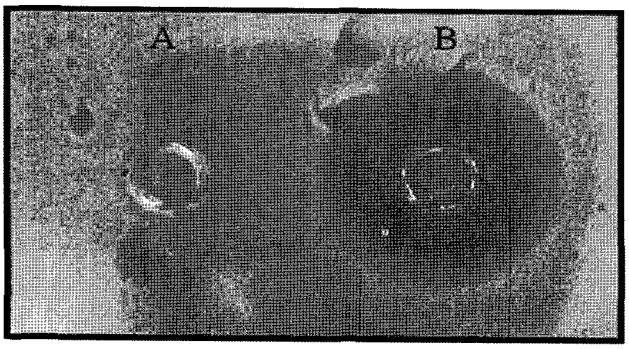


Fig. 5. Radial diffusion assay of the effect of MDCD-1L on the growth of *M. luteus*.
(A) Negative control; (B) purified MDCD-1L (200 μl of purified DCD-1L at 10 mg/ml, ~400 μmol).

Discussion

There is a growing interest among researchers in exploring better substitutes for antibiotics or their alternative use in medicine; therefore, antimicrobial peptides are receiving the appropriate attention [12, 16]. In order to find an alternative to chemical synthesis for the production of the DCD-1L peptide, we decided to use one of the sought-after systems for recombinant protein production involving *E. coli*. However, the use of an *E. coli* expression system for the production of AMPs may lead to toxicity toward host cells. In order to neutralize this effect, researchers have used thioredoxin, GST, inteins, *etc.* as fusion partners [8, 9]. In another approach, use of a large fusion partner coupled with the AMP can be considered. In this method, the fusion partner can be removed during the purification process and pure AMP can be obtained. Generally, while cleaving the recombinant protein from its fusion partner, chemical treatments using CNBr, formic acid, *etc.* following enzyme treatment with enterokinases or factor Xa are used. However, these methods involve the use of expensive enzymes, making them economically unfeasible. They carry the further risk of producing an AMP with low activity, which is worthless if it harbors target sites for enzymes or chemicals. A residual amino acid originating from the sequence between the target peptide and its fusion partner can also remain attached to the target peptide after cleavage.

Protein splicing converts precursors to biologically active proteins through a complex, self-catalyzed rearrangement [20]. In the past decade, mechanistic studies and extensive engineering of the naturally occurring protein splicing elements, termed inteins, have led to the development of a novel protein expression and purification system, named Intein Mediated Purification with an Affinity Chitin-binding Tag (IMPAC). This system uses an intein-mediated inducible self-cleavage to separate the target polypeptide from the CBD affinity tag, and in recent years, it has been widely used for polypeptide expression and purification from *E. coli* [5, 11]. This approach distinguishes itself from all other purification systems by its ability to purify in a single chromatographic step without the use of a protease. Therefore, in this work, we decided to use an intein fusion system to produce the DCD-1L target protein.

Initially, the native form of the DCD-1L peptide rather than MDCD-1L was expressed as an intein-mediated fusion protein (data not shown). The expression levels of the recombinant DCD-1L and MDCD-1L fusion proteins were similar, but there was no cleavage of the DCD-1L fusion protein. To solve this problem, we inserted an additional methionine residue between the intein and the DCD-1L junction to enhance DTT cleavage. Consequently, the recombinant MDCD-1L has an inauthentic amino acid residue at the N-terminus. At this time, we have not directly compared the antimicrobial activity of MDCD-1L versus native DCD-1L, but it seems that the additional methionine would have no effect [7].

In conclusion, inteins can be successfully used as fusion partners for the purification of MDCD-1L AMP by a single-step intein-mediated splicing. The intein-mediated fusion expression system is an alternative choice for the simple purification of small-sized peptides such as AMPs.

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