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A novel method for purifying recombinant human host defense cathelicidin LL-37 by utilizing its inherent property of aggregation

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Abstract

The importance of human LL-37 in host defense and innate immunity is well appreciated as reflected by an exponential increase of relevant literature in Pub-Med. Although several articles reported the expression and purification of this cathelicidin, some protocols suffered from low efficiency in enzyme cleavage of fusion proteins due to aggregation and poor separation of recombinant LL-37 from the carrier protein on reverse-phase HPLC. We present a new method for purifying LL-37 that avoids both problems. In this method, the fusion protein (a tetramer) purified by metal affinity chromatography was readily cleaved at a thrombin site 30-residue upstream of the LL-37 sequence. The released LL-37-containing fragment formed a large soluble aggregate (~95 kDa) at pH ~7, allowing a rapid and clean separation from the carrier thioredoxin (~14 kDa) by size-exclusion chromatography. Recombinant LL-37 was released from the isolated aggregate by chemical cleavage in 50% formic acid at 50 °C for 32 h. Due to a dramatic difference in retention time, recombinant LL-37 was well resolved from the S-Tag-containing peptide by RP-HPLC. Compared to previous procedures, the new method involves fewer steps and is highly reproducible. It increases peptide yield by 53%. NMR data support the aggregation of LL-37 into a tetramer with increase of pH as well as the feasibility of structural studies of an isotope-labeled antimicrobial peptide in the lipid micelle of dioctanoyl phosphatidylglycerol (D8PG) for the first time.

Keywords: Antimicrobial peptides; Cathelicidins; Chemical cleavage; Enzyme cleavage; *Escherichia coli*; FPLC; HPLC; LL-37; NMR; Protein aggregation

Human antimicrobial peptide LL-37 has attracted much research attention around the world since its discovery in 1995 [1]. This can be seen from an exponential increase of LL-37-related literature according to a Pub-Med search [2]. This 37-residue innate peptide is the only antimicrobial peptide in the cathelicidin family identified to date in humans [3]. Its protective effect to all humans against infection is well established. The lack of LL-37 in neutrophils can cause chronic periodontal diseases in patients with morbus Kostmann [4]. CRAMP (LL-37 analog) knockout mice are more susceptible to skin infection [5]. Further, expression of additional cathelicidins by gene therapy protects against bacterial infection [6]. In addition, LL-37 is also implicated in angiogenesis, wound closure, chemotaxis, signal transduction, and immunomodulation [3,7–10]. It is worthwhile to mention that the precursor protein (hCAP-18) of LL-37 in human seminal plasma can also be cleaved in vagina at an acidic pH by gastricsin into ALL-38, which contains one more alanine at the N terminus than LL-37 [11]. The in vivo post-translational processing of human LL-37 is further complicated by the recent discoveries of additional LL-37 derivatives in human sweat [12] and human skin [13]. Interestingly, the N-terminal truncated form of human LL-37 is capable of killing bacteria but loses its chemotaxis capability [13]. It is likely that the multiple functions of human LL-37 are encoded in different peptide regions.

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Our laboratory is interested in elucidating the structural basis of the multifunctional roles of LL-37. In our previous study, we determined the structures of several synthetic LL-37 fragments by two-dimensional NMR[1] [14]. Using TOCSY-trim, we identified a minimal antimicrobial and anticancer region corresponding to residues 17–29 of LL-37, which formed an amphipathic helix when bound to the micelles of either SDS or D8PG. Furthermore, a new peptide obtained by reversing the sequence of the minimal LL-37 antimicrobial region (the retro-peptide) is also active [15]. Of outstanding interest is that this retro-peptide derived from human host defense cathelicidin shows sequence homology to aurein 1.2, an antimicrobial and anticancer peptide isolated from the Australian Bell Frog [16], and a nontoxic *Escherichia coli* membrane anchor corresponding to the N-terminal sequence of glucose-specific enzyme IIA (IIA^{Glc}) [17–19], leading to a better understanding of all these peptides. However, an attempt to solve the structure of intact LL-37 in SDS micelles by classic 2D NMR methods turned out to be impractical owing to spectral overlap [14]. To facilitate structural studies of this peptide by multidimensional multinuclear NMR spectroscopy, we previously reported the cloning, expression and purification of isotope-labeled recombinant LL-37 [20]. In our original approach, a direct separation of LL-37 from the carrier protein thioredoxin on HPLC was not practical due to essentially identical retention times. To circumvent this obstacle, we introduced a second resin-binding step to get rid of the His-tagged carrier (or residual uncleaved His-tagged fusion protein) prior to HPLC purification. The second resin step is not trivial. The carrier protein might not be completely removed if the amount of resin added was underestimated. In another scenario, a portion of LL-37 might be lost as a result of non-specific binding to the resin if the amount of resin used was overestimated. In any case, either the quality or quantity of LL-37 could be compromised. In a subsequent LL-37 expression and purification paper [21], the GST fusion protein was only partially cleaved by factor Xa. Moreover, the LL-37 peak was poorly resolved from other peaks in the HPLC elution profile. All these problems caused a very low peptide yield (0.3 mg/L culture) in Ref. [21]. Thus, the separation of LL-37 after fusion protein cleavage by HPLC appears to be a common problem [20,21]. To solve this problem, we have developed a novel approach that makes use of the inherent property of LL-37 aggregation [22]. In this approach, the LL-37-containing aggregate is rapidly separated from the thioredoxin carrier by size-exclusion chromatography. The new approach described here is easy to perform and highly reproducible with excellent separation in all chromatographic steps.

[1] *Abbreviations used:* NMR, nuclear magnetic resonance; TOCSY, total correlated spectroscopy; PCR, polymerase chain reaction; LB medium, Luria-Bertani medium; IPTG, Isopropyl-β-D-thiogalactopyranoside; GST, Glutathione S-transferase; PBS, phosphate-buffered saline; FPLC, fast protein liquid chromatography; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; rLL-37, recombinant LL-37 with an additional Pro at the N terminus of the peptide; SDS, sodium dodecylsulfate; D8PG, dioctanoyl phosphatidylglycerol; CD, circular dichroism; HSQC, heteronuclear single-quantum coherence spectroscopy.

Materials and methods

Host strain *E. coli* BL21(DE3) and plasmid pET-32a(+) were purchased from Novagen. Restriction enzymes were obtained from New England Biolabs. PCR reagents and T4 DNA Ligase were purchased from Promega. QIAquick® PCR Purification Kit and QIAquick® Gel Extraction Kit were from Qiagen. All oligonucleotides were ordered from MWG Biotech. The composition of the minimal medium used for isotope labeling was previously described [20]. Formic acid (88%) was purchased from Fisher Scientific. Acrylamide, bisacrylamide, glycine, Tricine, and protein markers were purchased from Bio-Rad. Thrombin protease was purchased from Amersham Biosciences/GE. $^{15}NH_4Cl$ (>99% ^{15}N atom) was purchased from Spectra Stable Isotopes (Columbia, MD). Deuterated SDS and protonated D8PG were purchased from Cambridge Isotope and Avanti Lipids, respectively. Synthetic LL-37 (>95%), with a peptide sequence of LLGDFFRKSKEKIGKEFKRIVQRIKD FLRNLVPRTES, was purchased from Genemed Synthesis, Inc. (San Francisco, CA).

Expression vector construction

The vector hLL-37^{DP}/pET-32a(+) for the fusion expression of recombinant LL-37 was constructed as described [20]. This vector contains a thrombin site about 90 bases upstream of the AspPro formic acid cleavage site created previously by us. Between the thrombin and formic acid cleavage sites is the sequence coding for an S-Tag-containing peptide (Fig. 1a). To compare chemical and enzyme cleavage, we also created a new construct with two thrombin sites. The second thrombin site was generated in the forward primer to replace the original formic acid cleavage site (Fig. 1b). The forward primer was 5′-CGG*GGT ACC*CTGGTGCCACGCGGTTCTCTGCTGGGTGAT TTCTTCC GTAA-3′ whereas the reverse primer was 5′-GCG*GAATTC*CTAGGA CTCTGT-3′. The forward primer contains a recognition site for *Kpn*I (italicized) and a thrombin cleavage site (underlined) upstream of the coding sequence for LL-37 and the reverse primer contains a recognition site for *Eco*RI (italicized). PCR was performed as previously described using the original LL-37 expression vector as a template [20]. After purification, the amplified DNA was digested with *Kpn*I and *Eco*RI, and ligated into the pET-32a(+) plasmid. After transfection, the clone with the right insert was obtained based on the size of the expressed protein. The sequences of recombinant LL-37 and the two thrombin sites were