Optimized Methods for purification and NMR measurement of antibacterial peptide, bovine lactophoricin

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Abstract : Lactophoricin (LPcin-I) is a cationic amphipathic peptide with 23-mer peptide, and corresponds to the carboxy terminal 113–135 region of Component-3 of proteose-peptone. LPcin-I is a good candidate as a peptide antibiotic, because it has an antibacterial activity, but no hemolytic activity. On the other hand, its shorter analog (LPcin-II), which corresponds to the 119–135 region of PP3, has no antibacterial activity. In order to understand the structure-activity relationship under the membrane environments, we succeed to produce large amounts of LPcin-I and LPcin-II peptides. Peptides were overexpressed in the form of fusion protein in Escherichia coli, and purified with several chromatography techniques. In this paper, we introduce the optimizing processes of purification and NMR measurement.
Keywords : Bovine lactophoricin, Antibacterial peptide, Purification, NMR, HPLC

INTRODUCTION

The increasing resistance of classical antibiotics became already a serious health problem [1-3]. Recently, there are many kinds of proposed alternative strategies to develop a novel antibiotics. Among them, cationic amphipathic peptides come into the spotlight as a new generation antibiotics [2,4]. Several hundreds of antimicrobial peptides have been identified in diverse forms ranging from bacteria, fungi, plant, to mammals [5-7]. These peptides are alleged to kill target cells by disrupting the structure of cell membranes, but their mechanisms are not fully understood and more research is required on this topic.

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Lactophoricin (LPcin-I), corresponding to the carboxy terminal 113-135 region of Component-3 of proteose-peptone (PP3), is a cationic amphipathic peptide with 23-amino acid residues. LPcin-I is a good candidate as a peptide antibiotics due to the peptide is constrained the growth of both gram-negative and gram-positive bacteria, but has no hemolytic activity [8] . On the other hand, its shorter analogue (LPcin-II), which corresponds to the 119–135 region of PP3, has no antibacterial activity. It is interesting because LPcin-I and LPcin-II have similar charge ratios, identical hydrophobic/hydrophilic sectors [9-11], and interact with phospholipids. In order to understand the structure-activity relationship between two analogous peptides under the membrane environments, we cloned and expressed two recombinant peptides in Escherichia coli. We succeed to produce large amounts of LPcin-I and LPcin-II peptides through the optimized processes of expression and purification. Sometimes, purification of small peptides (<5kDa) still remains a difficult task. In our case, we expended a lot of time and effort on optimizing purification step of LPcin-I and LPcin-II peptides. We could get the initial structural data of LPcin-I and LPcin-II under the micelle condition by solution NMR spectroscopy [12] . Interestingly, spectral patterns of LPcin-I and LPcin-II peptides were quite different although the length of peptide sequence differing by only 6 residues in N-terminal region. NMR measurement condition was also optimized by varying pH or temperature of the micelle samples.

In this paper, we introduce the optimizing processes for the purification step and NMR measurement condition under the micelle environments. The methods and conditions used in this study, will be helpful to produce other antimicrobial peptides and/or small peptides and to perform the peptide structural studies by solution NMR spectroscopy.

MATERIALS AND METHODS

Expression and purification of fusion protein

A constructed vector for LPcin-I and LPcin-II were expressed in E.coli C41(DE3) cells with isotope-enriched M9 minimal media [12] . Ketosteroid isomerase (KSI)-fused protein was induced by addition of 1mM IPTG, and cells were grown for additional 16 hrs at 37 °C. Harvested cells were lysed by ultrasonication. Cell lysates were centrifuged and

the pellets were dissolved under denaturing conditions containing 6 M guanidine HCl for 5 hrs at room temperature, and then loaded on Ni-NTA column. After washing column with 4 volumes of washing buffer, KSI-fused protein was eluted with four volumes of elution buffer. To remove guanidine and salts, elutes were dialyzed against deionized water. The insoluble precipitates of KSI-fused proteins were collected and lyophilized.

Chemical cleavage and Dialysis

The freeze-dried fusion protein was dissolved in 70% formic acid, and chemically cleaved by cyanogen bromide for removing fusion partner KSI and His-tag. Reaction mixture was 5-fold diluted with water, and then dialyzed against deionized water to remove the impurities containing formic acids and cyanogen bromide. Dialysis was tested by using two different dialysis membrane with 500 and 1,000 MWCO. After dialysis tests, quantity and quality of LPcin-I and LPcin-II peptides were assessed by 12% tris-tricine PAGE. Finally, cleavage reaction mixture was lyophilized.

HPLC purification

LPcin-I and LPcin-II peptides were purified using preparative reversed-phase HPLC system on a C18 column (7.8 × 300 mm, Waters). Protein mixtures containing LPcin-II were dissolved in three different solutions, 5 %, 50 %, and 58 % (v/v) acetonitrile (ACN) in water containing 0.1 % (v/v) trifluoroacetic acid (TFA) at a concentration of 3mg/ml. The reaction mixture solution was filtered using 0.45µm syringe filter, and was then subjected to HPLC. HPLC seperation was monitored at 220 nm and 280 nm using a PDA detector with a linear gradient at a flow rate of 3 ml/min. Each fraction was pooled and identified by 12 % tris-tricine polyacrylamide gel electrophoresis (PAGE). The resultant LPcin-I and LPcin-II fractions were collected and lyophilized. The molecular weight and the purity were characterized by mass spectrometry.

Solution NMR spectroscopy

Micelle Samples were prepared by dissolving 1 mg of ^{15}N-labeled LPcin-I or LPcin-II peptides in 300L of 150 mM DPC-d38 containing 90 % H2O and 10 % D2O. Solution NMR experiments were performed on a Bruker AVANCE 800 spectrometer equipped with

a triple-resonance 5-mm probe with three-axis field gradients. NMR measurement conditions were optimized as increasing temperatures from 293 to 323 K in 5 degrees increments, and also changing pH values (4.0, 5.0, and 5.5). The two-dimensional $^1H/^{15}N$ HSQC spectra were acquired in phase-sensitive mode using the echo-antiecho method in the indirect dimension. Water resonance was suppressed using a water flip-back pulse and decoupling of ^{15}N was achieved by GARP4 modulation. Spectra were collected with 128–256 increments (8–16 scans each) in t1 and 1024 data points in t2 time domains, and data processing was performed using TOPSPIN 1.3 software (Bruker Biospin).

RESULTS AND DISCUSSION

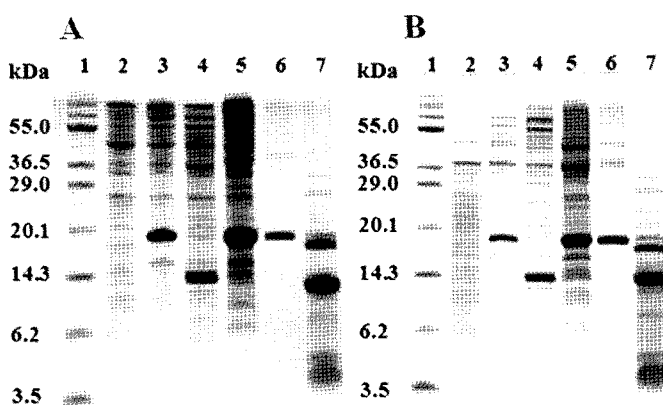### *Expression and purification of fusion protein*

Fig. 1. Tris-tricine (12%) PAGE diagrams showing the expression and purification of (A) LPcin-I and (B) LPcin-II. Lane 1, molecular weight marker; Lane 2, cells before IPTG induction; Lane 3, cells after IPTG induction; Lane 4, soluble fraction after lysis; Lane 5, insoluble fraction after lysis; Lane 6, KSI-fused protein purified from a Ni–NTA affinity chromatography; Lane 7, reaction mixtures after CNBr cleavage.
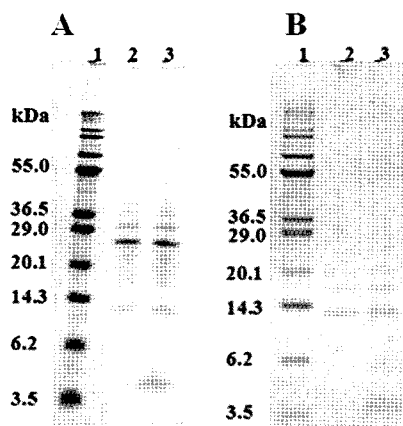
Fig. 2.   Tris-tricine (12%) PAGE diagrams of (A) LPcin-I and (B) LPcin-II after dialysis test. Lane 1, molecular weight marker; Lane 2, cleavage reaction mixture after dialysis using 1,000 MWCO membrane; Lane 3, cleavage reaction mixture after dialysis using 500 MWCO membrane.

High-level expression of KSI-LPcin-I-His6 (18.2 kDa) and of KSI-LPcin-II-His6 (17.5 kDa) was induced by addition of 1 mM IPTG (Fig. 1, lane 3). Since KSI-fused proteins were found in inclusion body (Fig. 1, lane 5) of cells grown at 37℃ in M9 medium enriched with stable isotopes, purification was performed from insoluble fractions. Insoluble fusion protein could be fully solubilized by unfolding with 6 M guanidine hydrochloride, and then was fully refolded by dialysis after Ni-NTA affinity chromatography. The total yield of purified fusion protein was about 200 mg per liter culture. Peptide purity and identities at each experimental setps were checked by 12% tris-tricine PAGE, as shown in Fig. 1.

Chemical cleavage and Dialysis

KSI-fused proteins were chemically cleaved with CNBr to release the target peptides, LPcin-I and LPcin-II (Fig. 1, lane 7). First, we tested by using 1,000 MWCO dialysis membrane. The majority of LPcin-I and LPcin-II peptides as well as impurities slipped from the dialysis membrane (Fig. 2, lane 2). When 500 MWCO dialysis membrane was used, LPcin-I (3.4 kDa) and LPcin-II (2.8 kDa) peptides were remained inside the membrane (Fig. 2, lane 3). But a little amount of peptides were left after a lyophilization. Finally, cleaved

reaction mixture was 10-fold diluted with water, and followed by lyophilization to maximize the protein content.
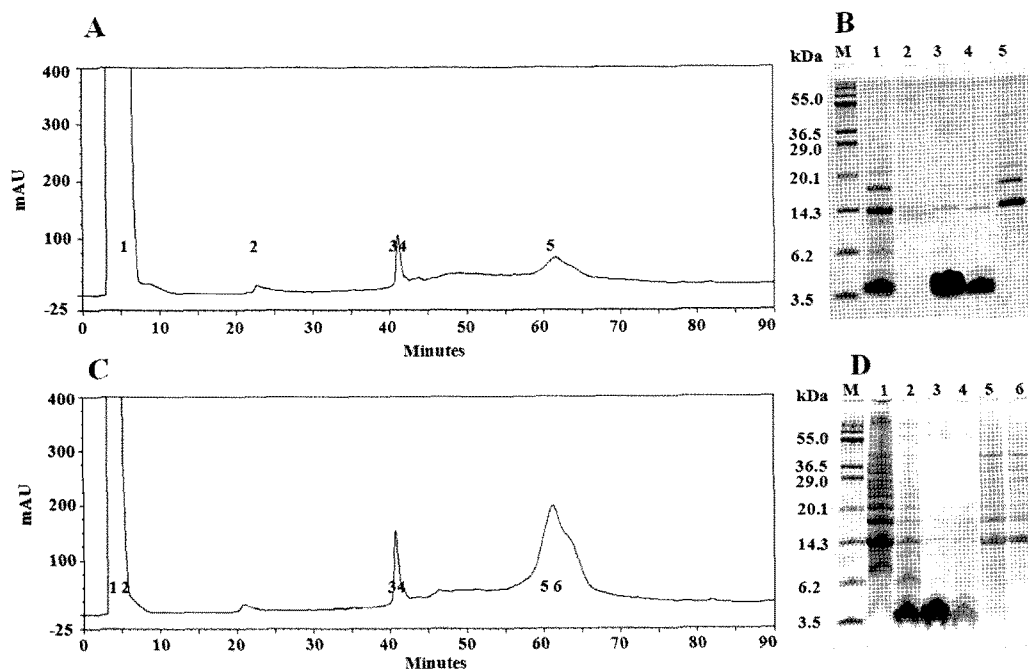
HPLC purification

Fig. 3. HPLC chromatogram of LPcin-II peptide dissolved in (A) 58 % ACN in water containing 0.1 % TFA, (C) 50 % ACN in water containing 0.1 % TFA, (E) 5 % ACN in water containing 0.1 % TFA. (G) HPLC chromatogram of LPcin-II peptide dissolved in 5 % ACN in water containing 0.1 % TFA. (B),(D),(F),(H) shows tris-tricine (12%) PAGE diagrams of HPLC fractions for (A),(C),(E),(G), respectively.

Cleaved reaction mixture was dissolved in three different solutions, 5 %, 50 %, and 58 % ACN in water containing 0.1 % TFA. For ACN concentration more than 58 %, the mixture was not clearly dissolved. Fig. 3A shows reversed phase HPLC chromatogram of LPcin-II sample dissolved in 58 % ACN in water containing 0.1 % TFA. From the result of
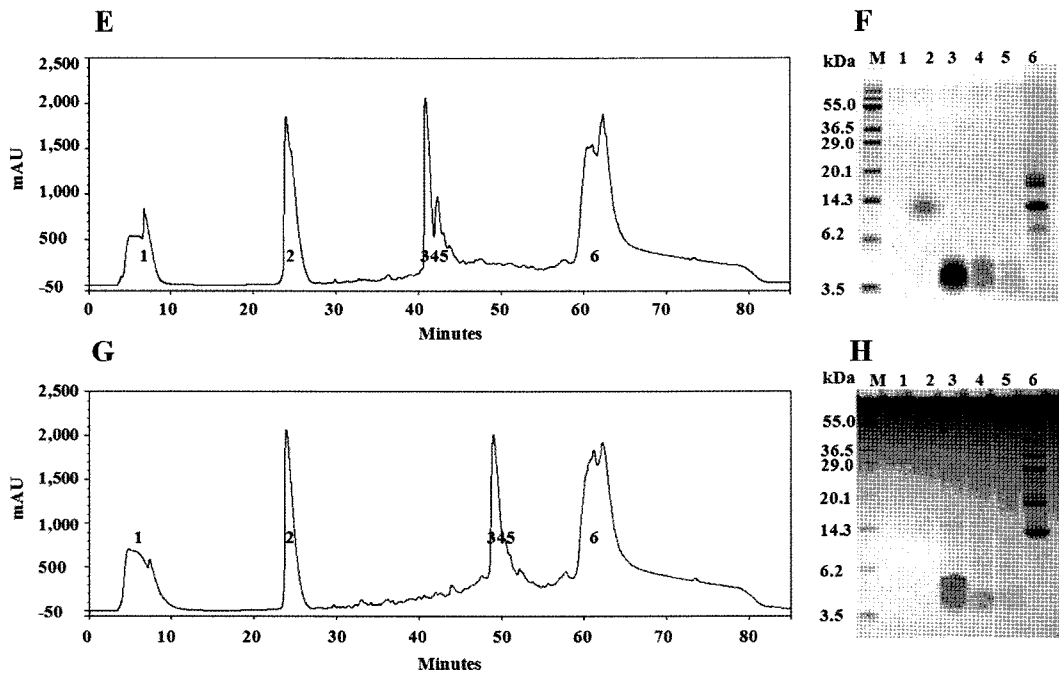
Fig. 4.   HPLC chromatogram of re-injected (A) LPcin-I and (C) LPcin-II peptide.   (B),(D) shows tris-tricine (12%) PAGE diagrams for HPLC fractions (A),(C), respectively.

Fig. 3B, LPcin-II peptide was eluted at 42 min (Fig. 3B, lane 3,4), but a lot of LPcin-II peptide was eluted in the initial void volume fraction with other impurities (Fig. 3B, lane 1). KSI fusion partner and incompletely cleaved fragments were eluted at 63 min (Fig. 3B, lane 5). Fig. 3C shows reversed phase HPLC chromatogram of LPcin-II sample dissolved in 50 % ACN in water containing 0.1 % TFA. In this case, the results were very similar to the results of former sample. Fig. 3E shows reversed phase HPLC chromatogram of LPcin-II sample dissolved in 5 % ACN in water containing 0.1 % TFA. From the result of Fig. 3F, LPcin-II peptide was eluted at 42 min (Fig. 3F, lane 3,4,5), and little of peptide was eluted in the initial void volume (Fig. 3F, lane 1). Major fraction containing LPcin-I and LPcin-II peptides in Fig. 3G and 3E) was collected and lyophilized. For better purified peptides, this main fraction was reinjected to HPLC and purified with a same condition (5 % ACN, 0.1% TFA). The 2nd HPLC choromatograms showed a single peak at 50min in Fig. 4A and 40min in Fig. 4C for LPcin-I and LPcin-II individually. The molecular weight and the

purity of the purified peptides were characterized by mass spectrometry. Fig. 5 shows the mass spectra of LPcin-I (Fig. 5A) and LPcin-II (Fig. 5B) obtained by HPLC purification. The measured molecular mass matched well with theoretical molecular weight of LPcin-I (3390.9 Da) and LPcin-II (2718.6 Da). Finally, we obtained higher purity and high yields of uniformly and selectively ^{15}N labeled LPcin-I and LPcin-II with 20~ 30 mg/L in M9 minimal medium.
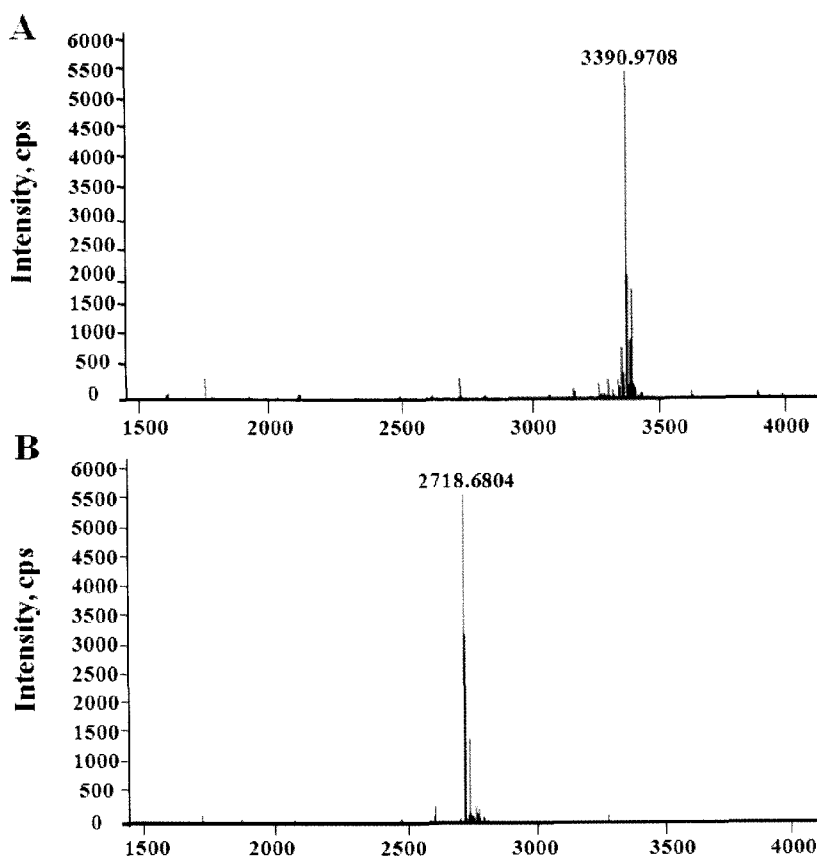

Fig. 5. Mass spectra of (A) LPcin-I and (B) LPcin-II obtained by HPLC purification. The measured molecular mass matched well with theoretical molecular weight of LPcin-I (3390.9 Da) and LPcin-II (2718.6 Da).

Solution NMR spectroscopy

Purified ^{15}N-labeled LPcin-I and LPcin-II peptides were characterized and compared using two-dimensional heteronuclear NMR spectroscopy to confirm the structural integrity and verify if the peptides were properly folded in DPC micelles or not. We optimized NMR measuring conditions by changing the pH value and temperature. Abnormal broadening or clustering of resonances is warning sign that the polypeptide is aggregated or improperly folded [13]. Fig. 6 shows HSQC spectra of LPcin-I measured at pH 4.0, 5.0, and 5.5. The spectral resolution was the best at pH 4.0 in Fig. 6A. Temperature was also optimized by acquiring HSQC spectrum from 293 to 323 K in 5 degrees increments. The spectral resolution was the best at 308 K for LPcin-I (Fig. 7D) and at 323 K for LPcin-II (Fig. 8D). The majority of the ^{1}H–^{15}N cross–peaks lies between 7.5 and 9.0 ppm. The 1.5 ppm dispersion of the amide ^{1}H chemical shifts is typical of ordered helical membrane proteins in micelles.
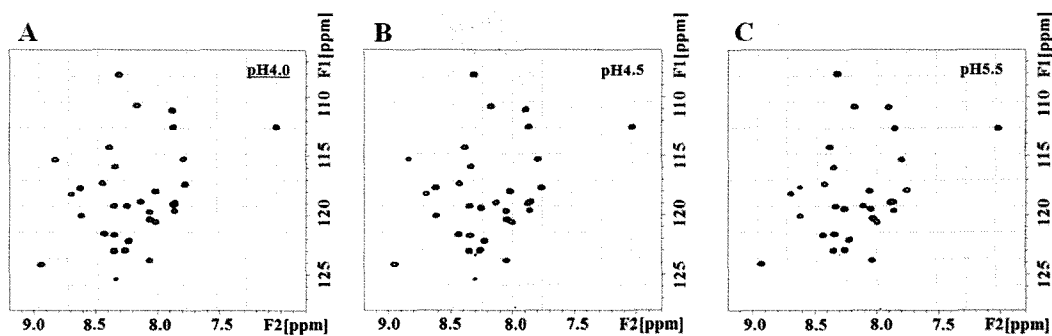

Fig. 6. ^{1}H/^{15}N 2D HSQC spectra of LPcin-I measured at (A) pH 4.0, (B) pH 5.0, and (C) pH 5.5.

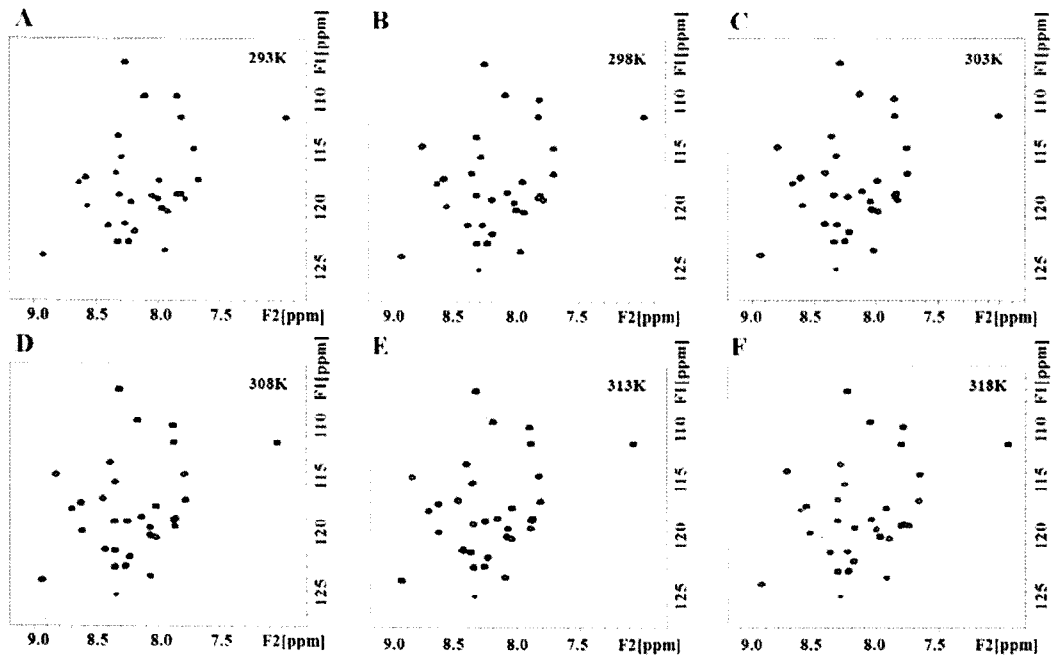Fig. 7. ^{1}H/^{15}N 2D HSQC spectra of LPcin-I measured at (A) 293 K, (B) 298 K, (C) 303 K, (D) 308 K, (E) 313 K, and (F) 318 K.
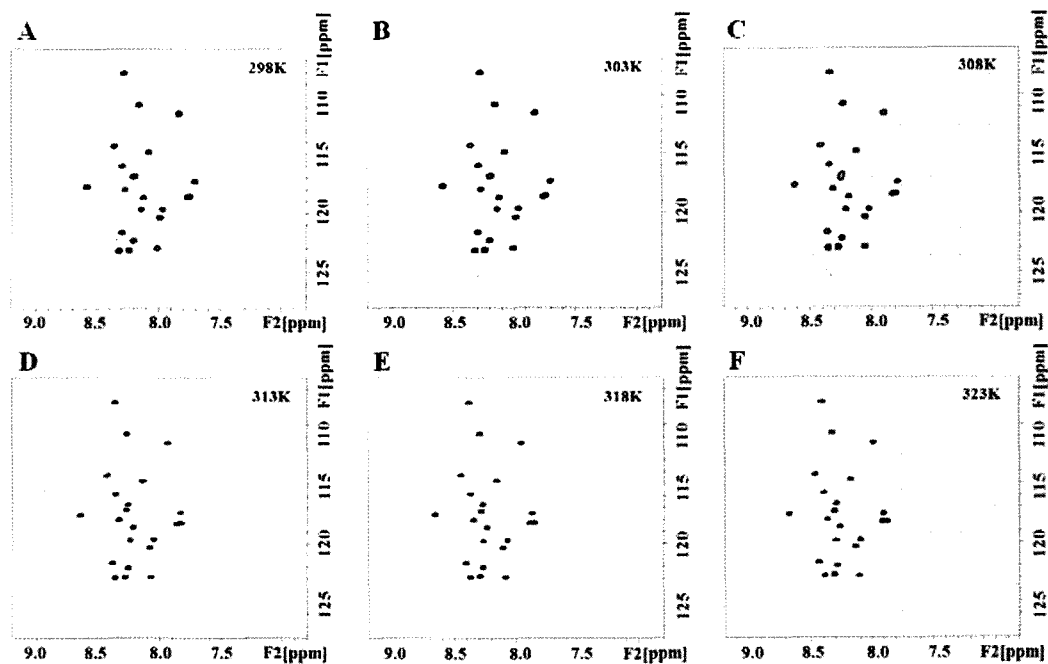
Fig. 8. $^1H/^{15}N$ 2D HSQC spectra of LPcin-II measured at (A) 298 K, (B) 303 K, (C) 308 K, (D) 313 K, (E) 318 K, and (F) 323 K.

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