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^{1}H, ^{15}N and ^{13}C Backbone Assignments and Secondary Structures of C-ter100 Domain of Vibrio Extracellular Metalloprotease Derived from *Vibrio vulnificus*

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Vibrio extracellular metalloprotease (vEP), secreted from *Vibrio vulnificus*, shows various proteolytic function such as prothrombin activation and fibrinolytic activities. Premature form of vEP has an N-terminal (nPP) and a C-terminal (C-ter100) region. The nPP and C-ter100 regions are autocleaved for the matured metalloprotease activity. It has been proposed that two regions play a key role in regulating enzymatic activity of vEP. Especially, C-ter100 has a regulatory function on proteolytic activity of vEP. C-ter100 domain has been cloned into the *E. coli* expression vectors, pET32a and pGEX 4T-1 with TEV protease cleavage site and purified using gel-filtration chromatography followed by affinity chromatography. To understand how C-ter100 modulates proteolytic activity of vEP, structural studies were performed by heteronuclar multi-dimensional NMR spectroscopy. Backbone ^{1}H, ^{15}N and ^{13}C resonances were assigned by data from standard triple resonance and HCCH-TOCSY experiments. The secondary structures of vEP C-ter100 were determined by TALOS+ and CSI software based on hydrogen/deuterium exchange. NMR data show that C-ter100 of vEP forms a β-barrel structure consisting of eight β-strands.

Key Words : Vibrio extracellular metalloprotease (vEP), Secondary structure, NMR, Cloning, Purification

Introduction

Extracellular proteases produced by pathogenic bacteria are considered to be important enzymes because of their implication in the pathogenesis. *Vibrio vulnificus* (*V. vulnificus*) is a gram-negative halophilic marine bacterium and is an opportunistic human pathogen causing wound infection and septicemia.[1-5] A metalloprotease secreted by *V. vulnificus* has many biological activities, including the activation of prothrombin[5] and the induction of vascular permeability through the generation of inflammatory mediators.[5-7] We have previously reported the enzymatic properties of a metalloprotease (vEP) secreted by *V. vulnificus* ATCC29307.[5] The vEP proenzyme is composed of a signal peptide, an N-terminal propeptide (so called nPP), an C-terminal region and a catalytic domain.[5,6] The proenzyme is activated through the removal of nPP domain,[6] which acts as an inhibitor as well as substrate for the cognate mature enzyme.[2,3,5] The processed active vEP enzyme (vEP-45) can be auto-processed further to form a 34 kDa enzyme (vEP-34), with the loss of C-terminal domain (C-ter100).[6]

vEP proteolytic activity assay in both absence and presence of C-ter100 region has been shown that no different enzyme activity on soluble proteins such as plasminogen and collagen IV. However, significant differences of proteolytic activity have been found on insoluble substrates such as fibrin and elastin, suggesting that C-ter100 region plays an important role in regulating proteolytic activity of vEP.[8,9] To determine detailed structural mechanism on the proteolytic function originated by propeptide, NMR and biochemical studies have been performed. Structural information in solution has been derived by NMR data combined with torsion angles from TALOS+ and CSI programs.[10,11] This data would be of essence in understanding its proteolytic function and it could be applicable to determine structural and functional properties of C-terminal propeptide domain of metalloprotease family proteins.

Materials and Methods

Cloning, Expression and Purification of vEP C-ter100. The vEP C-ter100 gene was obtained from Vibrio Vulnificus ATCC29307 genomic DNA by PCR amplification and subcloned into pET32a plasmid (Novagen Inc.) It was digested with BamHI and XhoI. Our construct contains Trx-tag/hexahistidine tag followed by TEV protease cleavage site (ENLYFQG). Cloning result was confirmed by DNA sequencing (Cosmogenetech.co). vEP C-ter100 (Lys 510-Tyr 609), including TEV cleavage sequence in N-terminal region, was cloned into pET32a which have His6 and thioredoxin. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3). Cell culture was performed in LB media with ampicillin (100 ug/mL) at 37 °C and the temperature was change to 15 °C after adding 50 uM isopropyl β-D-1-thiogalactopyranoside. The cells were grown for 15 h and harvested *via* centrifugation (6000 × g for 30

miniutes, 4). The cell pellet was resuspended and sonicated in lysis buffer consisting of 25 mM sodium phosphate (pH 7.0), 150 mM sodium chloride, 5 mM imidazole, 5 mM β-mercaptoethanol and protease inhibitor cocktail (Roche). Supernatant was separated by centrifugation (14000 × g for 30 minutes, 4 °C) and then passed through the His6-tag affinity Ni-NTA resin. Affinity column resin was washed twice out using washing buffer containing 40 mM imidazole. vEP C-ter100 peptide was eluted in elution buffer containing 500 mM imidazole. Eluted vEP C-ter100 was dialyzed to lysis buffer, then, thioredoxin and His6-tag was removed from target protein by treating TEV protease (1:1 molar ratio, 15 h at 25 °C), and vEP C-ter100 was reeluted in flow-through fraction during reverse phase column. To increase the protein purity, size exclusion gel chromatography by using HiLoad 16/60 Superdex^{TM} 75 column (Amersham Biosciences) was performed in NMR buffer, including 20 mM HEPES at pH 7.0, 100 mM sodium chloride, 10 mM DTT, 0.01% NaN_3.

Preparation of ^{15}N and ^{13}C/^{15}N labeled vEP C-ter100. To prepare ^{15}N or ^{13}C/^{15}N labeled protein, the cells were cultured in M9 media containing, $^{15}NH_4Cl$ (1 g/L) and/or ^{13}C-D-glucose (1 g/L) (Cambridge isotope Co.) for 20 h at 25 °C.[12] Purification procedure for isotope labeled protein is identical to that of described above. Final concentration of ^{15}N labeled and ^{13}C/^{15}N labeled C-ter100 is approximately 0.5-0.6 mM. ^{13}C/^{15}N labeled protein samples were prepared in two different buffer conditions containing 90% H_2O/10% D_2O for triple resonance experiments and 100% D_2O for H/D exchange experiment, respectively.

NMR Spectroscopy and Data Analysis. One-dimensional ^{1}H NMR, ^{1}H-^{15}N 2D-HSQC, and backbone NMR experiments such as HNCACB, CBCACONH, HNCA, HBHACONH and HNCO, were performed using a Bruker DRX 500 MHz spectrometer equipped with a pulse-field gradient triple-resonance probe at 298 K. 3D HCCH-TOCSY, HCC(CO)NH, ^{15}N-edited NOESY (τm = 150 ms) and ^{13}C-edited NOESY (τm = 150 ms) spectra were collected on a Bruker DRX 900 MHz spectrometer with Cryo-probe^{TM} at 298 K. 3D HCCH-TOCSY and ^{13}C-edited NOESY data were collected using NMR samples prepared in both 90%H_2O/10%D_2O and 100% D_2O solution for improving NMR sensitivity. WATERGATE pulse sequence was used for suppression of the solvent signal.[13] All collected spectra were processed and analyzed *via* XWINNMR (Bruker Instruments, Karlsruhe, Germany), NMRpipe/nmrDraw (Biosym/Molecular simulation, Inc. San Diego, CA, USA) software[14,15] and PINE-SPARKY program,[16] respectively.

Hydrogen/deuterium (H/D) Exchange NMR Experiments. For Hydrogen/deuterium exchange NMR experiment,

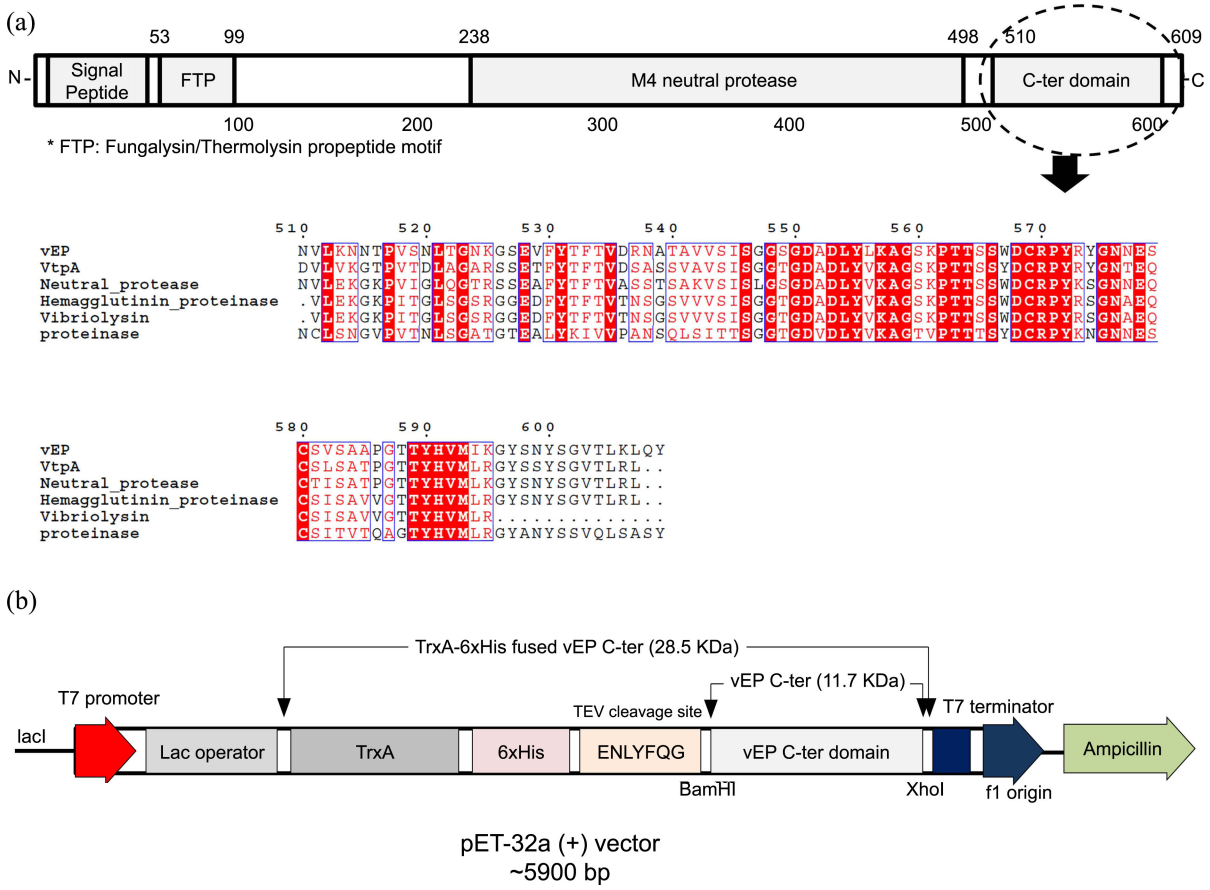

Figure 1. (a) Pair-wise alignment of vEP, VtpA, Neutral protease, hemagglutinin proteinase, vibriolysin and proteinase sequence. Pair-wise alignment was carried out using the CLUSTALW program (Thompson *et al*., 1994). Identical parts of sequences are shown by black letters and similar portions are shown in red box. (b) Recombinant vector map for the expression of vEP C-ter 100. The map of *E. coli* expression vector containing TrxA, 6xHis, TEV protease cleavage site and recombinant vEP C-ter was presented.

^{15}N lebeled vEP C-ter100 was lyophilized and dissolved in 100% D_2O solvent following quick dissolution method and it is pre-incubated for 24 h at 4 °C. A series of ^{1}H-^{15}N 2D HSQC spectrum were collected and slowly exchanging amide resonances were identified.

Results and Discussion

Plasmid Construct and Purification of vEP C-ter100. Data from primary sequence alignment identifies evolutionarily or structurally related positions in a collection of amino acid sequences (Fig. 1(a)). The vEP gene was obtained from *Vibrio Vulnificus* genomic DNA by PCR amplification and successfully subcloned into pET32a (Novagen Inc.) at the BamHI and XhoI site (Fig. 1(b)). The construct contains a Trx/hexahistidine with a TEV protease recognition sequence (ENLYFQG) in the N-terminal extension. vEP C-ter100 protein was successfully expressed in both 250 mL LB and M9 media using 1 mM IPTG at 25 ºC. After Ni-NTA affinity chromatography, vEP C-ter100 shows an excellent solubility. Fusion protein was eluted around at the imidazole concentration of 500 mM and purified as a purity of > 90% determined by SDS-PAGE (Fig. 2(a)). The target protein, vEP C-ter100, was obtained through TEV treatment followed by Ni-NTA column work (Fig. 2(a)). The size of fusion protein is determined as a 27 kDa of molecular weight and target protein is about 10 kDa, respectively. To get vEP C-ter100 with higher purity (> 95%), size exclusion chromatography was performed using HiLoad 16/60 superdex^{TM}75 (Fig. 2(b)). The final yields of C-ter100 protein was calculated as ~10 mg per 500 mL of LB media with purity of 95% (Fig. 2(a)). One-dimensional ^{1}H NMR spectrum was used to check sample condition (Fig. 3(a)).

NMR Resonance Assignments. 2D [^{1}H-^{15}N]-HSQC spectrum (Fig. 3(b)) demonstrated that most of backbone NH resonances were well spread over the spectrum. The backbone sequential resonance assignments of vEP C-ter100 were completed by data from triple resonance experiments.[17] Most of geminal amide protons were also completely assigned. Side chain assignments were performed by a combined use of HCC(CO)NH, HCCH-TOCSY, ^{15}N-edited NOESY spectra. Side chains of aromatic residues were assigned using ^{13}C-edited NOESY. Some of aromatic sidechain could not be assigned completely due to ambiguity of resonances. Summary of backbone and side chain assignments was deposited to the BioMagnetic Resonance Bank (BMRB.-http://www.bmrb.wisc.edu) under accession number 18338. Slow exchange rates of amide protons depend on the hydrogen bonding interaction or burial degree of amide proton.

Hydrogen/deuterium (H/D) Exchange NMR Experiment. From the hydrogen/deuterium exchange experiment, we identified the residues participated in hydrogen bonds or located in buried core. About 40% of amide proton resonances were observed after 24 h incubation time with D_2O solution (Fig. 3(c)). Most of residues are located on the secondary structure, however some resides, for example G523 and Y600 are located on the loop region. This data supports that C-ter100 region of vEP forms a very stable and compact structure although it is a C-terminal domain of vEP. This observation agrees well with secondary structures determined by NMR data. Slowly exchanging amide protons also correlate with hydrogen bonding or buried region inside the protein core proposed previously.[18,19] Since asparagine residues are hydrophilic, those are mostly solvent exposed

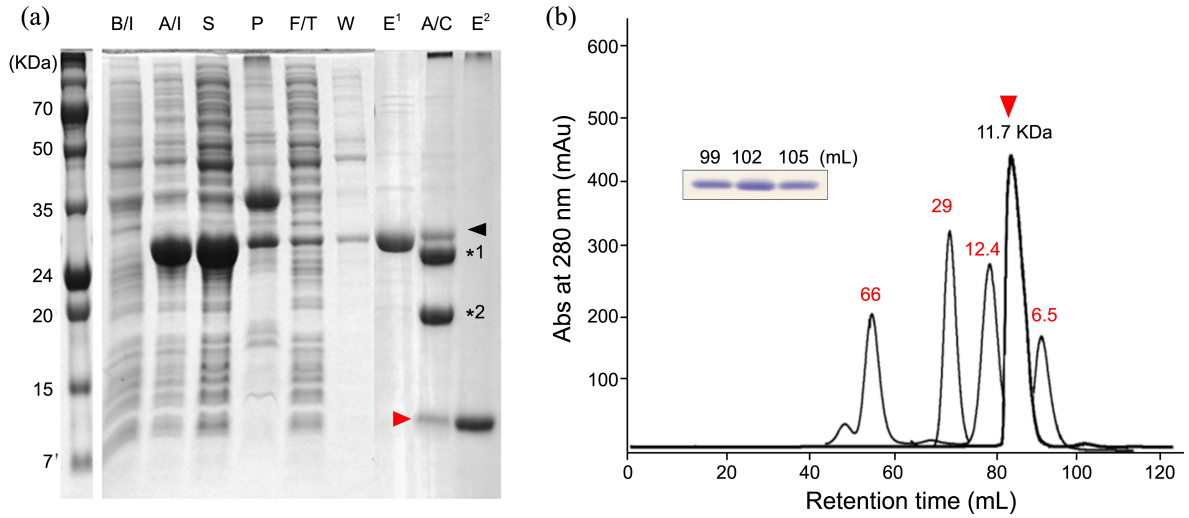

Figure 2. Data from SDS-PAGE and size exclusion chromatography demonstrate solubility, purify and molecular weight of vEP C-ter100. (a) SDS-PAGE analyses of purified vEP C-ter100. Molecular weight of fusion protein is determined as 28.5kDa and target protein is about 11.7 kDa. The molecular weight markers are appended to indicate the size of the protein in each lane. Lane 1 and 2, whole cell lysate before and after IPTG induction; lane 3 and 4, supernatant and pellet of cell lysate, respectively; lane5, flowthrough; lane 6, 60 mM washing; lane 7, fusion protein elution; lane8, after TEV cleavage; lane 9, cleaved target protein elution in reverse column work. (b) Elution profile of the size exclusion chromatography. The standard marker proteins were already analyzed by Origin 7.01. vEP C-ter100 was eluted from 99 to 105 mL. The highest elute fraction was detected in 102 ml. Molecular weight of vEP C-ter100 was calculated by the equation (logy = –0.0253x + 3.2038, R^2 = 0.9915, x = Elution fraction).

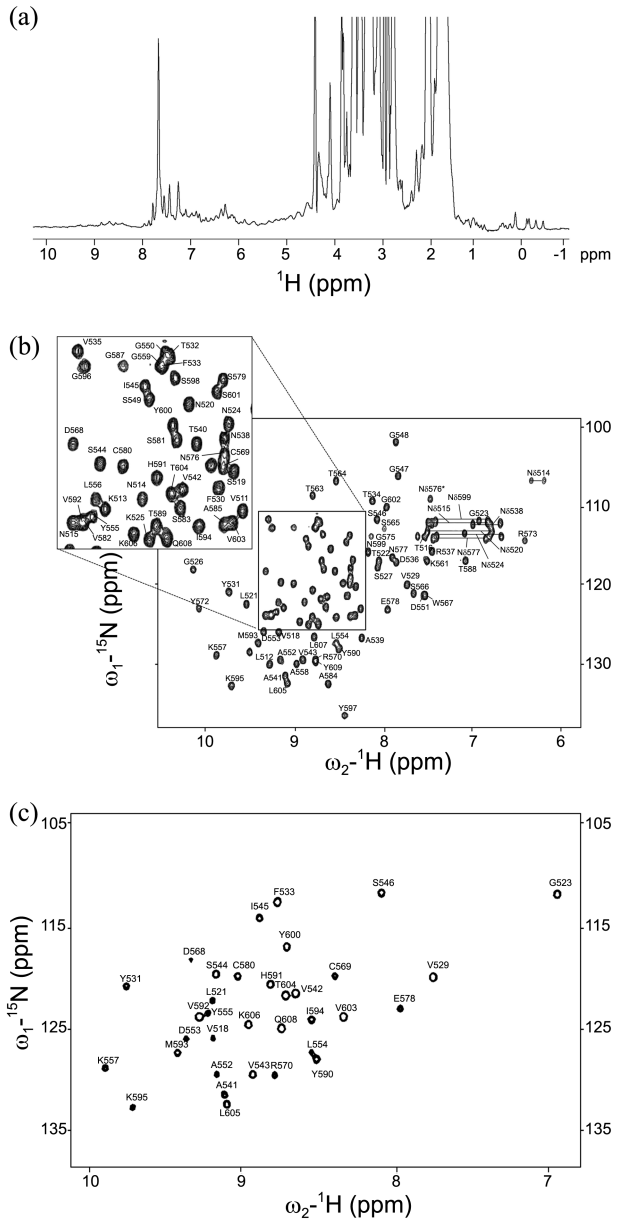

Figure 3. NMR spectrum of vEP C-ter100 (a) one-dimensional ^{1}H NMR spectrum (b) 500 MHz ^{1}H/^{15}N Heteronuclear single quantum correlation (HSQC) spectrum of ^{15}N-labeled vEP C-ter100 at pH 7.0 and 298 K. Side-chain amide protons from Asn were indicated by lines. The geminal protons of N576 side chain was revealed different intensities and one of the geminal amide protons (marked by *) was clearly shown in the HSQC spectrum. (C) ^{1}H/^{15}N Heteronuclear single quantum correlation (HSQC) spectrum of the hydrogen/deuterium (H/D) exchange experiment. Slowly exchanging amide protons are detected after 24 h when protein was dissolved in D_2O solution.

and found on molecular surface. vEP C-ter100 has nine asparagine residues and most of their amide proton signals were not shown except N572, which might be involved in hydrogen bonding (Fig. 3(c)). The hydrogen/deuterium exchange data agrees with secondary structures determined by chemical shift indices (CSI) (Fig. 4(a)).

Secondary Structures of vEP C-ter100. The secondary structures of vEP C-ter100 were determined by CSI and

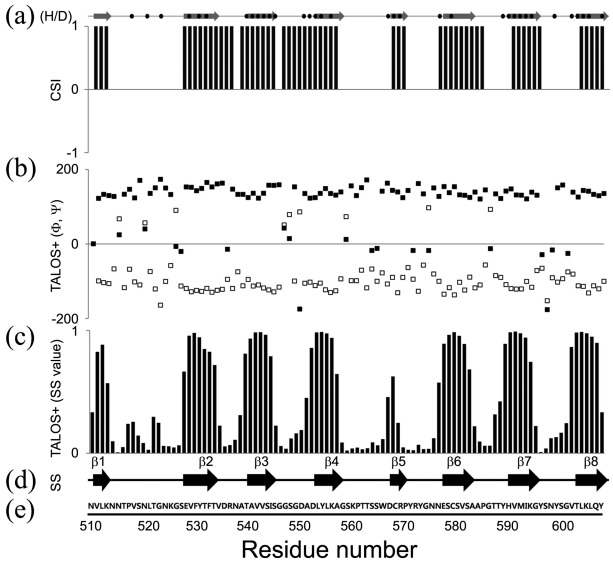

Figure 4. (a) Secondary structures derived from CSI and NH/ND exchange data. Slow exchange residues are marked by filled circles on upper CSI plot. (b), (c) Plots of PHI (Φ) and PSI (Ψ) value indicated backbone dihedral angles and secondary structures determined from TALOS+. (d) Consensus secondary structure predicted by CSI and TALOS+. (e) Amino acid sequence of vEP C-ter100 region.

TALOS+ based on NMR data (Fig. 4). Chemical shifts of HA, CA, CB and CO atoms together with backbone chemical shifts were served as an input data to calculate torsion angle values. The secondary structures determined by NMR data are well agreed with those of all other experimental data such as ^3JHN coupling constants, backbone amid-proton exchange and chemical shift index values. All together, our data suggest that vEP in solution has a compact β-sheet topology with eight β-strands.

Conclusion

Vibrio extracellular metalloprotease (vEP), secreted from *Vibrio vulnificus*, demonstrated various proteolytic function including prothrombin activation and fibrinolytic activities. C-terminal domain of vEP, which consisted of 100 residues of C-terminal region of vEP, has been successfully cloned into the *E. coli* expression vectors, pET32a and pGEX 4T-1 with TEV protease cleavage site and purified using gel-filtration chromatography. vEP C-ter100 was isolated with high purity (> 95%) and determined as a monomeric form. Isotope-labeled (^{15}N/^{13}C) C-ter100 was prepared for NMR studies. Based on NMR data, we could conclude that vEP C-ter100 exists as an independent domain and it forms a compact β-sheet structure with eight β-strands.

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