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High-yield Expression and Characterization of Syndecan-4 Extracellular, Transmembrane and Cytoplasmic Domains

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The syndecan family consists of four transmembrane heparan sulfate proteoglycans present in most cell types and each syndecan shares a common structure containing a heparan sulfate modified extracellular domain, a single transmembrane domain and a C-terminal cytoplasmic domain. To get a better understanding of the mechanism and function of syndecan-4 which is one of the syndecan family, it is crucial to investigate its three-dimensional structure. Unfortunately, it is difficult to prepare the peptide because it is membrane-bound protein that transverses the lipid bilayer of the cell membrane. Here, we optimize the expression, purification, and characterization of transmembrane, cytoplasmic and short extracellular domains of syndecan4 (syndecan-4 eTC). Syndecan-4 eTC was successfully obtained with high purity and yield from the M9 medium. The structural information of syndecan-4 eTC was investigated by MALDI-TOF mass (MS) spectrometry, circular dichroism (CD) spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy. It was confirmed that syndecan-4 eTC had an α-helical multimeric structure like transmembrane domain of syndecan-4 (syndecan-4 TM) in membrane environments.

Key Words : Syndecan, Syndecan-4, Transmembrane, NMR spectroscopy, Membrane protein

Introduction

The syndecan family consists of heparan sulfate proteoglycans, which are present on the surface of all cell types in humans. This family regulates cell-to-cell interaction, cell adhesion, cell proliferation and angiogenesis, as well as helps in healing wounds by activation of growth factors.[1-5] All types of the syndecan family are anchored in the cell plasma membrane and their structures are subdivided into three major sections, namely extracellular (ecto-), transmembrane (TM) and cytoplasmic (cyto-) domains. Syndecan-1 and syndecan-4 of this family have an interesting feature associated with tumor progression and metastasis: they were overexpressed on the cell surface during development of tumor cell.[6-16] Their roles in tumorigenesis have not identified clearly, and their behaviors towards tumor cell have been shown to be different. An overexpression of syndecan-1 is related to tumor aggressiveness and poor prognosis.[6,16,17] But the overexpression of syndecan-4 is stimulated by tumor suppression molecule and revert anti-adhesion by tenascin-C which is well known to block cell adhesion and enhance tumor proliferation.[18,19] Syndecan-4 enhances focal adhesion and reduces cell motility at the ectodomain of this protein, which was mediated by the cytoplasmic domain that binds with activated protein kinase C-α (PKC-α) and PI-4,5-biphosphate (PIP_2).[6,20-22] In other words, the structural alteration of the transmembrane and cytoplasmic domains by binding with PKC-α and PIP_2 regulates the function of the ectodomain.

The cytoplasmic domain of syndecan family is divided largely three region, C1, V and C2 domain.[6] The C1 and C2 domain of syndecan-4 have highly conserved sequence with other family, but V domain has distinguished sequence from other family and this region leads specific function of syndecan-4. The binding of PIP_2 at V domain of syndecan-4 causes the structural change of whole cytoplasmic domain of sydnecan-4 and this leads binding and activation of PKC-α.[23,24] This property was concerned to researchers, thus it had been well-known that structure of cytodomain of syndecan-4 was formed to multimer and this structural property dominated these functions.[25-27] However, this structural information was obtained only with cytoplasmic domain of syndecan-4 without other regions like transmembrane domain and ecto domain. Thus, we tried to study the more realistic structural studies of syndecan-4 that contain transmembrane, cytoplasmic, and ectoplasmic domain.

To understand this behavior of syndecan-4 in a tumor cell, it will be necessary to identify the structural alteration of TM, cyto- and short ecto- domains in syndecan4 (syndecan-4 eTC), but the structural study of syndecan-4 eTC has been limited because the membrane protein had been difficult to express and purify due to its insufficient yield and low solubility. And it also requires specific environments in regard to the cell membrane to investigate the original structure of the membrane protein. Hence, earlier structural studies on syndecan-4 were only performed for the cytoplasmic domain and the ectodomain, excluding the transmembrane domain.[28-31] To solve these problems, we optimized the expression and purification of syndecan-4 containing the transmembrane domain and introduced a structural study using NMR spectroscopy that can be performed under a membrane-like environment such as micelle or bicelle. We

previously reported the successful expression and purification of syndecan-4 transmembrane domain (syndecan-4 TM) and structural studies using NMR spectroscopy.[32] In this study, we tried to express and purify syndecan-4 eTC, which consists of the transmembrane domain (25 aa), the cytoplasmic domain (28 aa), and a slight ectodomain (4 aa) to investigate the function and structure of the syndecan-4 more clearly. We obtain high yield of protein (10 mg/L of M9 culture), it is sufficient for the structural study using NMR spectroscopy and investigate preliminary structural information using the samples in membrane environments.

Experimental

Cloning of Recombinant Syndecan-4 eTC in pET31b(+) Vector. Two complementary oligonucleotide sequences with 174-bases correspond to syndecan-4 eTC chemically synthesized in IDT (Integrated DNA Technology, USA). The two oligonucleotides were annealed at 95 ºC, and the annealed DNAs were identified and purified by 2% agarose gel elecetrophoresis. Purified DNA after gel extraction was ligated into the AlwN I digested pET 31b(+) vector system (Novagen, USA) since the cohesive ends of the synthetic oligonucleotides were compatible with the A1wN I site of this vector system. In order to identify the inserted DNA, the vector system for the expression of syndecan-4 eTC was transformed to a *NovaBlue* competent cell (Novagen, USA), which is a cloning host cell. Transformed host cell was incubated overnight at 37 ºC and the inserted DNA was purified. The purified plasmid DNA was digested by XbaI and XhoI (New England Biolabs, USA) and identified by insertion of a fusion protein gene in plasmid DNA by 1.5% agarose gel. To optimize the expression condition of syndecan-4 eTC, the purified plasmid DNA was transformed into three kinds of *Escherichia coli* (*E. coli*) strains: BL21(DE3)pLysS(Novagen, USA), C41(DE3) and C43 (DE3)(Avidis, France). All expression host cells were plated on the LB agar plate with carbenicillin (50 mg/mL) and were incubated overnight at 37 ºC. The incubated colony of single cells was collected on a plate and was dissolved in 5 mL LB medium with carbenicillin and was incubated overnight at 37 ºC. 50 mL of incubated cell culture was dissolved and incubated in 5 mL LB media with carbenicillin. When the OD_{600} of the cell culture reached at 0.5, the expression of fusion protein, KSI-syndecan-4 eTC-His_6-tag, was induced by 1 mM IPTG (isopropyl-β D-thiogalactopyranoside). The expression of the fusion protein was identified by 14% tris-tricine SDS PAGE, and the C41(DE3) cell strain was selected for large scale expression of syndecan-4 eTC.

Expression and Purification of KSI-fused Syndecan-4 eTC. To obtain uniformly ^{15}N labeled syndecan-4 eTC for NMR structural study, an expression host cell was incubated in M9 minimal medium with $(^{15}NH_4)_2SO_4$ and was induced by 1 mM IPTG of OD_{600} of 0.5. Induced fusion protein was harvested into a pellet by centrifugation (6000 RPM, 4 ºC, 30 min). The collected pellet was lysed by lysozyme (0.5 mg/mL) and ultrasonication, and the fusion protein was collected into a pellet by centrifugation (13200 RPM, 4 ºC, 30 min). Fusion protein with His_6-tag was purified from the cell mixture by Ni-NTA immobilized metal affinity chromatography (IMAC). NTA resin was charged to Ni^{2+} ion by Ni charging buffer that was made to 50 mM $NiSO_4 \cdot 6H_2O$ and unbounded Ni ion was removed by 100 mL ddH2O. Protein mixture was resolved in binding buffer (20 mM Tris, 500 mM NaCl, 5 mM Imidazole and 6 M Guanidine-HCl, pH 7.9-8.0) and also Ni charged resin calibrated by this buffer. After applied protein mixture at IMAC, washing buffer (20 mM Tris, 500 mM NaCl, 16 mM Imidazole, 6 M Guanidine-HCl, pH 7.9-8.0) was used to remove proteins that was unbounded with Ni charged resin. KSI fused syndecan-4 eTC was eluted from resin by eluting buffer (20 mM Tris, 500 mM NaCl, 500 mM Imidazole, 6 M Guanidine-HCl, pH 7.9-8.0) Purified protein was identified by 12% tris-tricine SDS PAGE. To remove denaturing agents, salts and other impurities, the fusion protein mixture was dialyzed with 10,000 MWCO membrane tubing and obtained to precipitate. The fusion protein was collected with lyophilization for further step. Syndecan-4 eTC was isolated from the fusion partner protein, KSI and His_6-tag, by chemical cleavage. The lyophilized fusion protein was dissolved in 70% formic acid (5 mg/mL, Fluka, USA) and was reacted with CNBr (50 mg/mL, Sigma-aldrich, USA) in dark for 5 h. The mixture of syndecan-4 eTC and fusion partner protein was dialyzed with 1,000 MWCO membrane tubing in deionized distilled water to remove CNBr and formic acid. The protein mixture solution was lyophilized after dialysis.

To isolate syndecan-4 eTC, protein mixtures were purified by a semi-preparative reversed-phase HPLC column (Waters Delta Pak C4, 300 × 7.8 mm, USA) on a Delta 600 HPLC system (Waters, USA). The lyophilized protein mixtures were dissolved in 1:4 HFIP/MC during 1 h. The sample was centrifuged to remove undissolved impurities (15000 RPM, 4 ºC, 30 min). Eluent A contained in 2% ACN/3% IPA/95% H_2O/0.1% TFA and eluent B contained in 28% ACN/47% IPA/20% TFE/5% H_2O/0.1% TFA. The gradient profile from 0% eluent A to 100% eluent B at a flow rate of 3 mL/min for 95 min was used. The peak fractions of HPLC were detected at 220 and 280 nm using photo diode array (PDA) detector, respectively. The peak fractions were identified by 12% Tris-tricine SDS PAGE.

Mass Spectrometry of Syndecan-4 eTC. The purity and the molecular weight of syndecan-4 eTC were identified by matrix-associated laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Syndecan-4 eTC was dissolved in 300 μL of 30% ACN/0.1% TFA, and 12 μL aliquot of the peptide solution was mixed with a matrix of α-Cyano-4-hydroxycinnamic acid (CHCA), and the final concentration of the syndecan-4 eTC peptide was 1.4 pmol. MALDI-TOF mass spectrometer (AB SCIEX 4800 *plus* MALDI TOF/TOF™ Analyzer (AB SCIEX, USA)) was used in the reflector mode for higher resolution and used in the linear mode for high mass molecules. The CID gas at tandem mass spectrometry of syndecan-4 was used to air to perform finger print of syndecan-4 eTC and the collision energy was

used to 2 kV.

CD Spectroscopy of Syndecan-4 eTC. The structure of syndecan-4 eTC was roughly identified by CD spectroscopy. All CD experiments were performed by Jasco J715 spectropolarimeter (Jasco, Japan). A sample container was used with a 1 mm path-length quartz cuvette. All spectra of the CD experiments were measured between 190 and 260 nm with resolution of 0.2 nm. A sample was prepared so that syndecan-4 eTC could be dissolved in a 10 mM sodium phosphate buffer and added with 0-100 mM dodecylphosphochline (DPC) at pH 4.0. To correct the baseline of all spectra, the spectrum of the buffer without the peptide was subtracted to all final spectra. The CD signal Ψ (mdeg) was converted to mean residue molar ellipticity, θ, in degree cm^2/dmol

NMR Spectroscopy of Syndecan-4 eTC in DPC Micelles. NMR measurement of Syndecan-4 eTC was carried out by using a triple resonance X, Y, Z-gradient TXI probe in a Bruker Avance 800 MHz spectrometer (Bruker Biospin, Germany). Uniformly ^{15}N labeled syndecan-4 eTC peptide of 2 mg was dissolved in a 300 μL 90% H_2O/10% D_2O with 100 mM perdeuterated DPC-d_{38}, which contained 10 mM Na_2HPO_4, 40 mM DTT, and 1 mM NaN_3. The experimental condition was optimized at 323 K and pH 4.0. Two dimensional ^1H-^{15}N heteronuclear single quantum coherence (HSQC) spectra observe correlation between ^1H and ^{15}N of the syndecan-4 eTC peptide. ^{15}N-edited two dimensional heteronuclear multiple quantum coherence-nuclear Overhauser enhancement spectroscopy (HMQC-NOESY) was obtained for the uniformly ^{15}N labeled syndecan-4 eTC. All spectra were obtained in 32 scans in 1024 (f2) and 256 increments (f1). 300 ms mixing time for HMQC-NOESY spectroscopy was optimized and used. All spectra were obtained and processed in Bruker Topspin 2.1 (Bruker Biospin, Germany)

Results and Discussion

Construction of Expression Vector. The vector system was used for expression of the gene for syndecan-4 eTC (174 bps), as shown in Figure 1. The DNA sequence of syndecan-4 eTC with the gene of ketosteroid isomerase(KSI) at downstream and hexa-histidine tag at upstream was inserted into the vector. KSI corresponding to the 125 amino acids is the expression fusion partner, and the hexa-histidine tag was added for purification using Ni-NTA IMAC. The recombinant plasmid with DNA of syndecan-4 eTC was transformd by the *Novablue*, cloning host cell. To identify the insertion of the DNA sequence of the KSI fused syndecan-4 eTC into the pET31b(+) vector, the cloned plasmid in the host cell was purified and digested by the restriction enzymes XhoI and XbaI. Digested DNA fragments were identified by 1.5% agarose gel electrophoresis (data not shown). The sequence correctness of the KSI fused protein was verified by automated DNA sequencing. To optimize the expression level and condition of syndecan-4 eTC, the recombinant plasmid was transformed into three different kinds of *E. coli* strains

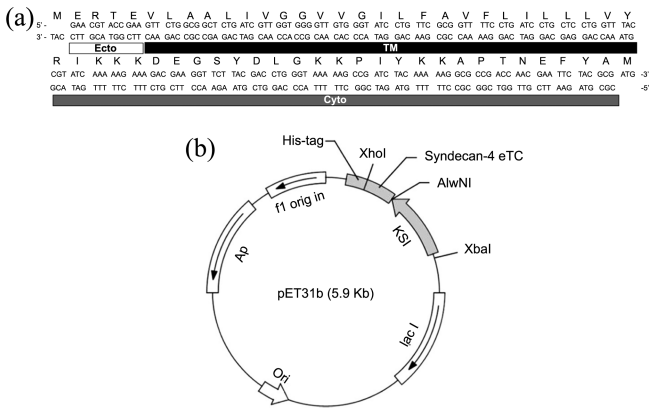

Figure 1. (a) DNA oligomer and amino acid sequence of Syndecan-4 eTC. (b) Schematic diagram of expression vector system for KSI fused syndecan-4 eTC.

for selection of the expression host cell with the highest expression level. And the C41(DE3) host cell was selected for the expression of KSI fused syndecan-4 eTC. We also optimized the incubating temperature, growth time, and induction OD by IPTG in the M9 minimal medium.

Expression and Purification of KSI-fused Syndecan-4 eTC. To obtain a large amount of protein for structural analysis using NMR spectroscopy, the KSI fused protein was expressed in M9 minimal medium, which enabled uniform selection of the ^{15}N labeled protein. KSI fusion protein was induced by 1 mM IPTG at $O.D._{600}$ of 0.5 at 37 °C and was incubated 16 h after induction. The expression level of the fusion protein was identified by 12% tris-tricine polyacrylamide gel electrophoresis (PAGE), and the induced protein in C41 (DE3) cell line was presented to a broad smear band which is a typical band type of transmembrane protein from 21 kDa to 34 kDa, which was corresponded to the KSI-(syndecan-4 eTC)-His_6 fusion protein (Figure 2, lane 3, 5-6).

To collect the KSI fused protein, the harvested cell was dissolved in a lysis buffer and was lysed by using an ultrasonicator. The target protein after centrifugation was deposited in a pellet (Figure 2, lane 5), and the supernatant of the cell lysate contain lysozyme (approx. 14 kDa), which lysed the cell membrane (Figure 2, lane 4). The KSI fused protein was purified by Ni-NTA immobilized metal affinity chromatography using His_6-tag, and the major component of the target protein was purified, as shown in lane 6 in Figure 2. To remove guanidine-HCl and salts in the KSI-fused protein, the eluent after Ni-NTA affinity chromatography was dialyzed with 10 kDa MWCO membrane tubing in deionized water for overnight. After dialysis, the fusion protein was obtained by precipitation, and the white powder of the fusion protein was collected by lyophilization.

The syndecan-4 eTC was released from the fusion protein, fusion partner KSI, and Hig_6-tag by CNBr chemical cleavage in 70% formic acid for 5 h. The protein mixture in 70% formic acid was diluted to 5 fold deionized water, was dialyzed with 1 kDa MWCO membrane tubing in deionized water overnight and was collected by lyophilization. As

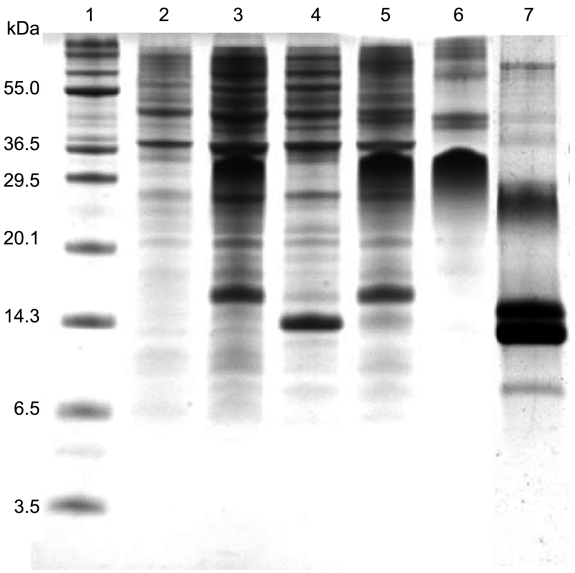

Figure 2. 12% Tris-tricine PAGE analysis of KSI fused syndecan-4 eTC after expression and purification. Lanes 1: molecular weight marker. Lane 2: cells before induction by IPTG, Lane 3: cells after 16 h growth after induction by IPTG, Lane 4: supernatant after cell lysis, Lane 5: pellet after cell lysis, Lane 6: KSI fused sydencan-4 eTC after Ni-NTA immobilized metal affinity chromatography and Lane 7: mixture of syndecan-4 eTC and KSI after CNBr chemical cleavage.

shown in lane 7 in Figure 2, the cleavage of fusion protein was identified by the presence of isolated KSI (13.4 kDa). In this result, bands of syndecan-4 eTC predict that present to 6.4 kDa (monomer) and 13 kDa (dimer) but actually present approx. 7.5 kDa (monomer) and 15 kDa (dimer) in SDS PAGE. This result was caused by properties of transmembrane that bound SDS micelle randomly formed α-helix and random coil. Also, maximum SDS-binding level was 1.4 g/g (SDS/polypeptide) in soluble protein but was 0.7-4.5 g/g (SDS/polypeptide) in membrane protein. Bands of membrane protein in SDS PAGE generally were represented to excess mass larger than original mass since these properties lead to slightly decrease mobility of these.[33-35]

Finally, the protein mixture of 150 mg was obtained in 1 L M9 minimal medium growth. The frozen and dried protein mixture was dissolved with bath sonication in 1:4 HFIP/MC solution during 1 h for HPLC purification. To prevent sample heating, the protein mixture solution was sonicated in an ice bath. To remove insoluble impurities, the protein mixture solution was centrifuged for 30 min and injected to the reverse phase C-18 column of the semi-preparative HPLC system. The protein mixture was detected at 220 and 280 nm by the PDA detector and was identified by 12% tris-tricine PAGE. The major component of syndecan-4 eTC was eluted at 67 min (Figure 3(a) and 3(b), peak number 5). In Figure 3(b), the syndecan-4 eTC(6.5 kDa) band of 5 did not correspond to its molecular weight, because syndecan-4 eTC formed dimeric conformations as sydecan-4 TM did.[32] Also, fraction 6 and 7 after HPLC purification presented some trace of protein on SDS-PAGE, but there is little after lyophilization for collection of target protein.

Identification of Syndecan-4 eTC. The purity for syndecan-4 eTC after HPLC isolation was reconfirmed that major peaks in HPLC chromatogram were analyzed by MALDI-TOF mass spectrometry. In the spectra of fraction 5, two peaks were observed at *m/z* 3229.29 and *m/z* 6458.04, as shown in Figure 4(a), and minor peak at *m/z* 3229.29 corresponded to the 2+ ionic charges for the non-isotope labeled syndecan-4 eTC, the peak at *m/z* 6458.04 corresponded to the molecular ion of the monomeric form. In this result, measured values were well matched to a theoretical mass of syndecan-4 eTC (M^{2+}: *m/z* 3228.4, molecular ion: *m/z* 6456.7). To obtain the finger print region of syndecan-4 eTC, tandem mass spectrometry with precursor ion at *m/z* 6458.04 was proceeded, but it was not occurred the fragmentation of syndecan-4 eTC in finger print region when was used with 1 kV collision energy and with air CID gas. The 2 kV collision energy was used because the higher collision energy to obtain higher internal energy of precursor ions needed to generate the product ion of this protein in finger print region. And we could obtain tandem mass spectrum of syndecan-4 eTC as Figure 4(c). The result of *de novo* sequencing in Figure 4(d) presented that it is consistent with b-NH_3 ion of 8 residues (-RTEVLAAL-) in syndecan-4 eTC. This presents that syndecan-4 eTC was successfully

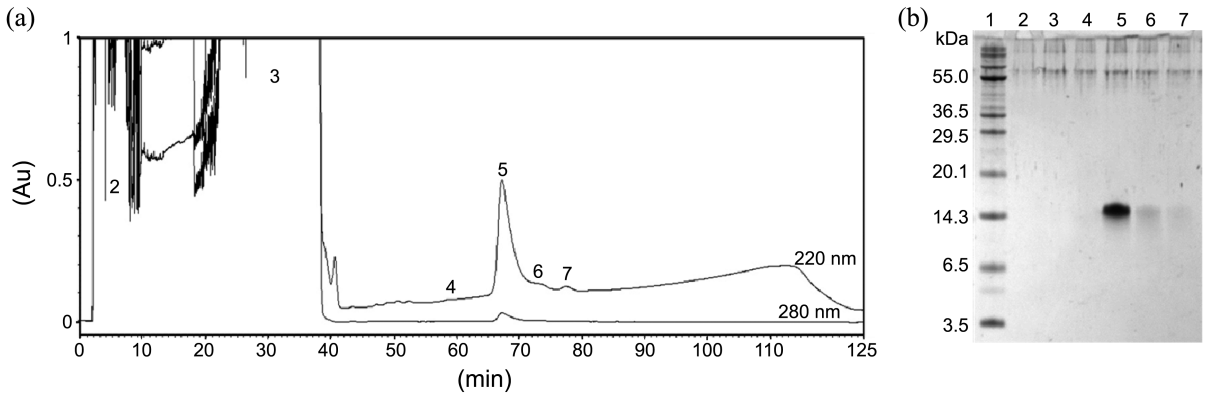

Figure 3. (a) Semi-preparative reversed phase HPLC chromatogram of Syndecan-4 eTC detected at 220 and 280 nm with PDA detector. (b) 14% Tris-tricine PAGE analysis of HPLC peak fraction.

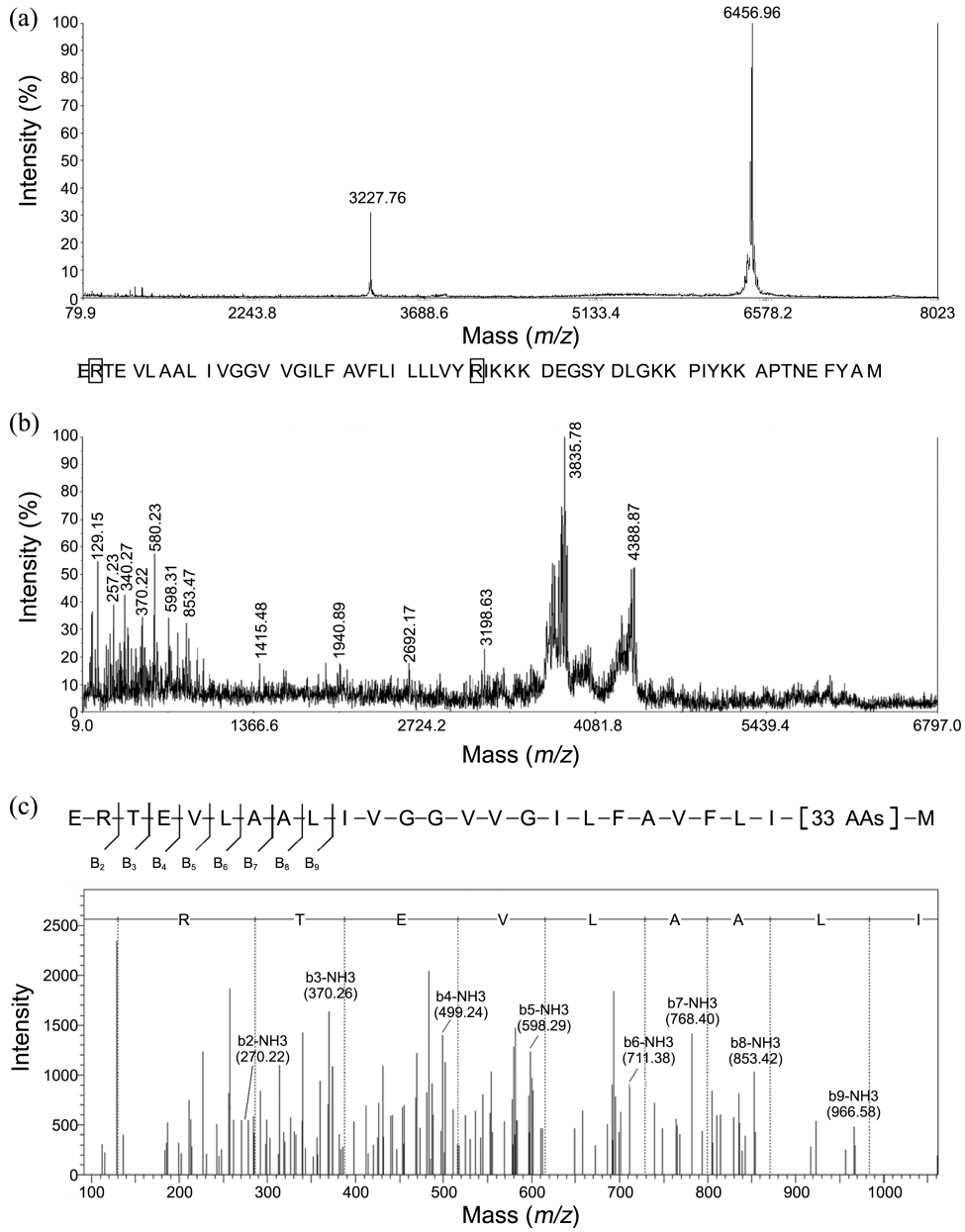

Figure 4. (a) MALDI TOF MS spectrum of non-isotope labeled Syndecan-4 eTC after reverse phase HPLC purification (fraction 7 of HPLC chromatogram) obtained by reflector mode. Syndecan-4 eTC with the molecular ion appears at *m/z* 6458.04 and that with ion charges of 2+ present at *m/z* 3229.29. At this spectrum, the presence of M^{2+} ion are easily formed by (b) two arginine (pI = 10.76) of syndecan-4 eTC (PI = 9.35). (c) Reflector mode MS-MS spectra of syndecan-4 eTC with 2 kV collision energy. The collision gas was used to air. (d) The result of *de novo* sequencing of syndecan-4 eTC.

obtained to high purity using HPLC purification.

Finally, fraction 5 was selected for further structural studies and final yield of syndecan-4 eTC was 10 mg per 1 L M9 minimal media.

Structural Elucidation of Syndecan-4 eTC. The structural study of syndecan-4 eTC in membrane environments was accomplished by CD spectroscopy and NMR spectroscopy. The secondary structure of syndecan-4 eTC was analyzed by CD spectroscopy from 190 nm to 260 nm. The structural change of this protein in different membrane environments was observed using DPC micelle: syndecan-4 eTC without DPC micelle was in random coil structure and was changed to α-helical structure by adding of DPC micelle, as shown in Figure 5. Syndecan-4 eTC had very stable α-helical structure at 100 mM DPC.

The structure of syndecan-4 eTC in DPC micelles was also observed by using 800 MHz solution NMR spectroscopy. The 2D ^{1}H-^{15}N HSQC experiments of syndecan-4 eTC were optimized and carried out at 313 K and pH 4.0. ^{1}H-^{15}N correlation peaks of the syndecan-4 eTC in a HSQC spectrum were represented for the amide proton of each amino acid, as shown in Figure 6. All cross-peaks were well dispersed and the two or more resonance pairs of ASN side chain at 112 ppm were presented. This result means that the

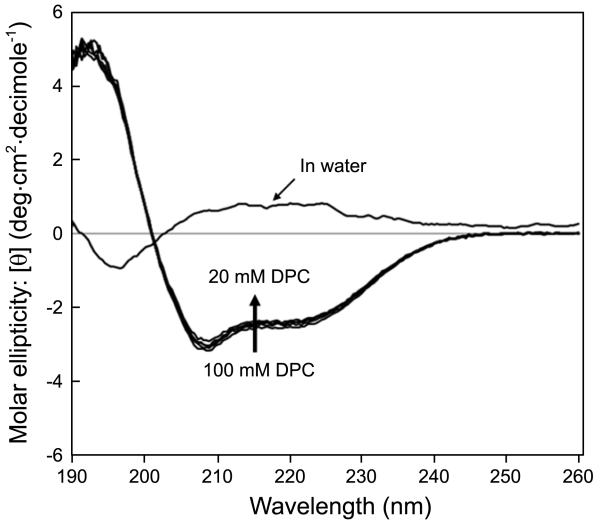

Figure 5. Circular Dichroism spectra of Syndecan-4 eTC. Structure of sydecan-4 eTC was varied with the concentration of detergent.

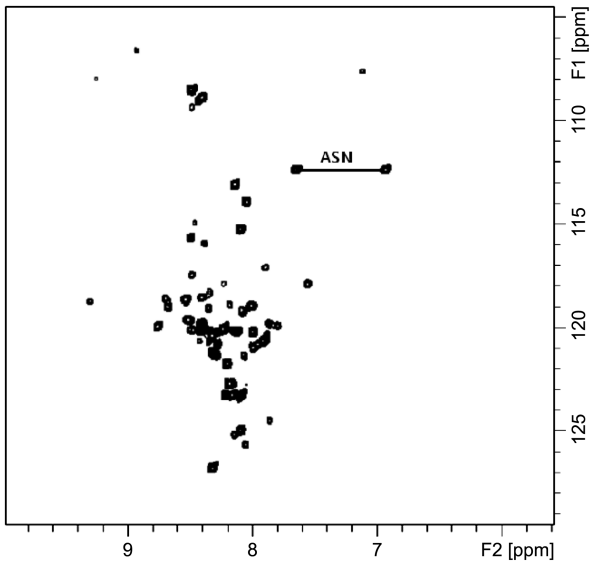

Figure 6. ^{1}H-^{15}N HSQC spectra of uniformly ^{15}N labeled syndecan-4 eTC. This spectrum was recorded with 1 mM of syndencan-4 eTC in 100 mM DPC-d_{38} at 323 K and pH 4.0 by using 800 MHz Avance solution NMR (Bruker Biospin, Germany).

structure of syndecan-4 eTC was mainly α-helical structure and might have multimeric conformations in membrane environments as syndencan-4 TM did in DPC micelles.[32]

Conclusion

We constructed the cloning system for the expression of syndecan-4 eTC, which has the cytoplasmic domain, transmembrane domain, and short extracellular domain. Syndecan-4 eTC was successfully expressed at high yield by an optimized purification and isolation process. The purified syndecan-4 eTC was retained sufficiently for further structural studies using solid-state NMR spectroscopy.

MALDI-TOF MS, CD, and solution NMR study of syndecan-4 eTC in DPC micelle showed that syndecan-4 eTC might form dimeric conformation. The property of oligomerization of syndecan-4 was suggested and reported by many researchers. Also, this property of sydnecan-4 was reported that formation of oligomeric structure of sydnecan-4 dominated basic function, binding of PKC-α and α-actin, in human body.[25,36-38] Dimerization of transmembrane domain of syndecan-4 was well known but studies for these were generally proceeded by modification of transmembrane domain or binding with fusion partner, because it was very difficult that membrane protein with transmembrane region expressed and purified without fusion partner.[39] But, we successfully have carry out for syndecan-4 eTC that contained transmembrane, ecto- and cyto- domain without other protein.

As mentioned above, the result of this study was in good agreement with the formation of dimerization of syndecan-4 TM that was previously reported.[32] These results will provide important clues that can elucidate the multimerization of syndecan-4 that binds with PKC-α. Also, further structural studies using solution and solid-state NMR spectroscopy will investigate the native structure of syndecan-4 eTC in a membrane-like environment to elucidate the function of syndecan-4 in a cell membrane.

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