Ubiquitin Fusion System for Recombinant Peptide Expression and Purification: Application to the Cytoplasmic Domain of Syndecan-4

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The cytoplasmic domain of syndecan-4, a type I transmembrane heparan sulfate proteoglycan, was over-expressed as a fused form with the ubiquitin molecule in *Escherichia coli*, and the fusion protein was purified using immobilized metal affinity chromatography (IMAC). The cytoplasmic domain was released from its fusion partner by using yeast ubiquitin hydrolase (YUH), and subsequently purified by reverse phase chromatography. The integrity of the resulting peptide fragment was checked by MALDI-TOF and NMR spectroscopy. The yield of the peptide was 3.0-1.5 mg per liter in LB or minimal medium, respectively. The recombinant expression and purification of this domain will enable us its structural and functional studies using multidimensional NMR spectroscopy.

Key Words : Ubiquitin fusion, Syndecan-4, MALDI-TOF, NMR

Introduction

The syndecans belong to a family of type I transmembrane heparan sulfate proteoglycan (HSPG), and they play an important role in the regulation of various cellular processes.[1-3] Four types of mammalian syndecans have been reported so far. Among these, syndecan-4 is believed to play a critical role in the assembly of focal adhesions with microfilament bundles.[4,5] For example, fibronectin (FN) interacts with β1 integrins and syndecan-4 which are the cell-surface receptors.[3-5] During initial cell attachment and spreading, β1 integrins primarily regulate the cell adhesion, but later on, the binding of FN with syndecan-4 is an essential step for the assembly of focal adhesions and actin stress fibers.[4,5]

There are three distinct regions in the cytoplasmic domain of syndecans.[3] C1 region lies right next to the transmembrane domain, then the V region, and the C2 region is at the carboxy terminus. The C1 and C2 regions are highly homologous. The C2 region of all four mammalian syndecans can interact with several PDZ domain-containing proteins like syntenin,[6] CASK,[7] or GIPC (synectin/SEMCAP-1).[8] These interactions may play a role in trafficking and/or establishing networks of submembranous signaling complexes.[9,10] The V region, flanked by C1 and C2 domains, is unique to each syndecan. The V region of syndecan-4 can interact with phosphatidylinositol 4,5 bisphosphate (PIP_2) and the catalytic domain of the protein kinase Cα.[11-15]

The three dimensional structures of the cytoplasmic domain of syndecan-4 were solved by NMR spectroscopy.[16,17] However, it would be much more meaningful if the structure of the whole complex is known. To get the complex structure, a large amount of sample is needed, and for NMR spectroscopy, it also needs to be labeled, which requires the recombinant expression of the peptide in a suitable host. Here we present our method of producing and purifying recombinant cytoplasmic domain of syndecan-4 by using the ubiquitin fusion system in *Escherichia coli*.

Materials and Methods

Construction of the 4L expression plasmid with $(His)_6$-tagged ubiquitin as a fusion partner. The gene coding for 4L was chemically synthesized by CoreBio, Inc. (Seoul, Korea). The amino acid sequence of 4L is RMKKKDEGSY DLGKKPIYKK APTNEFYA (28mer), and the nucleotide sequence was obtained by reverse translation. The sense strand (93mer) was 5'- gat ccc gca tga aga aga agg atg aag gca gtt acg act tgg gca aga aac cca tct aca aaa aag ccc cca cca acg agt tct acg cat gac -3', and the antisense (93mer), 5'- tcg agt cat gcg tag aac tcg ttg gtg ggg gct ttt ttg tag atg ggt ttc ttg ccc aag tcg taa ctg cct tca tcc ttc ttc ttc atg cgg -3'. Two strands were annealed by heating at 95 °C for 5 min, and slowly cooling down to the ambient temperature. Upon annealing, the N- and C-termini became compatible with BamHI and SalI ends respectively. The plasmid pET-28a/ubi[18] was digested with BamHI and SalI, purified, and ligated with the synthetic syndecan 4L gene. The resulting plasmid was named pET-28a/ubi/4L (Fig. 1).

Expression and purification of ubiquitin-4L fusion protein from an LB medium. The pET-28a/ubi/4L plasmid was brought into the expression host, Rosetta(DE3)pLysS (Novagen, Madison, WI). One colony was used to inoculate a 100 mL LB medium supplemented with 50 μg/mL kanamycin and 34 μg/mL chloramphenicol, and grown overnight in a 37 °C shaking incubator. The fully grown culture was mixed with a fresh one liter LB medium with the same antibiotics cocktail the next morning. The culture was grown at 37 °C, and IPTG was added to a final concentration

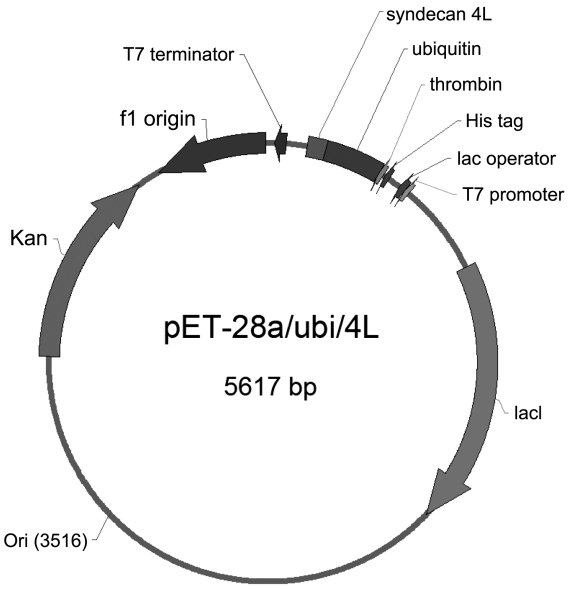

Figure 1. Plasmid vector map of pET-28a/ubi/4L.

of 0.5 mM when the OD_{600} reached 1.0. The culture was harvested 5 hours later and the cells were resuspended in 30 mL of 10 mM TrisHCl pH 8.0. The cells were lysed by freeze-and-thaw and the DNA was fragmented by ultrasonication. The soluble fraction was retained after centrifugation at 15,000 rpm for 20 min, and loaded onto HiTrap Chelating HP column (5 mL). The column was put on the ÄKTA Prime system (GE Healthcare, Piscataway, NJ, USA), and imidazole gradient of 0 to 0.4 M was applied. The fractions containing ubiquitin-4L were collected and pooled into one tube. The amount of protein in the pooled fractions was measured by using Protein Assay kit (Bio-Rad, Hercules, CA, USA).

Expression and purification of ubiquitin-4L fusion protein from a minimal medium. Rosetta(DE3)pLysS containing the pET-28a/ubi/4L was grown at 37 °C in a 5 mL LB medium inoculated from a single colony. 1 mL of the fully grown culture was mixed with 100 mL of the minimal medium and grown overnight at 37 °C. The fully grown culture was mixed with a 0.9 liter minimal medium and the culture was grown at 37 °C. For the uniform [^{15}N]-labeling, 0.1 g of $^{15}NH_4Cl$ was provided as a sole nitrogen source for 100 mL culture. The production and purification steps were the same as the previous section

Purification of 4L. To the purified ubiquitin-4L fusion protein, β-mercaptoethanol and YUH were added to the final concentrations of 1 mM and 2 mg/mL, respectively. The mixture was incubated at 37 °C for one hour. The reaction mixture was directly loaded onto a Resource RPC column (GE Healthcare, Piscataway, NJ, USA), and an acetonitrile gradient of 15 to 40% was applied using the ÄKTA Basic system. The 4L fraction was pooled and lyophilized. The final product was checked by MALDI-TOF. The peptide concentration of the pooled fractions was calculated from the theoretical extinction coefficient, ε_{280} = 1.295 $(mg/mL)^{-1}cm^{-1}$.

NMR experiments. The NMR sample contained 0.05 mM [^{15}N]-4L in 10 mM sodium phosphate buffer, pH 7.0 and 10% D_2O. The ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectrum was collected at 10 °C on a Bruker AvanceII 500 MHz spectrometer. The raw data contained 2048 and 256 complex points in t_2 and of t_1, respectively. The data was processed using NMRPipe software package.[19] The final spectrum contained 1024 and 256 real points in t_2 and of t_1, respectively.

Results and Discussion

Construction of expression plasmids. The genes coding for YUH and ubiquitin-4L were inserted into His-Tag containing vectors to facilitate the purification of the desired proteins. The pET-28a/ubi/4L plasmid had the kanamycin resistance rather than ampicillin, and we could grow the cells for a longer period of time after the IPTG induction. The ubiquitin fusion system was chosen according to the work done by Kohno *et al.*[20] One of the most widely used fusion partner for producing proteins is the glutathione S-transferase (GST), but the size of GST is 26.2 kDa, which is considerably larger than 8.5 kDa of ubiquitin. The relatively smaller size of ubiquitin gives the advantage of higher yield of the target protein when the total expression levels of the fusion proteins are not much different.

Expression and purification of ubiquitin-4L fusion protein. The expressed proteins appeared as 3 bands whose sizes corresponded to ubiquitin alone, ubiquitin with partial 4L, and ubiquitin with full-sized 4L as can be seen in lane 7 of Figure 2. The band corresponding to ubiquitin with partial 4L appeared to be the weakest, and the other two bands were of about the same intensity. We suspect that some protease in the *E. coli* cell was in action, and this kind of unwanted cleavage was reported in other previous studies.[18,21] The purity of the fusion protein was examined by SDS-PAGE as shown in Figure 2. The yield of the fusion protein was around 24 or 15 mg per liter of LB or minimal medium, respectively. The yield of the final peptide was 3.0-1.5 mg per liter in LB or minimal medium, respectively. However,

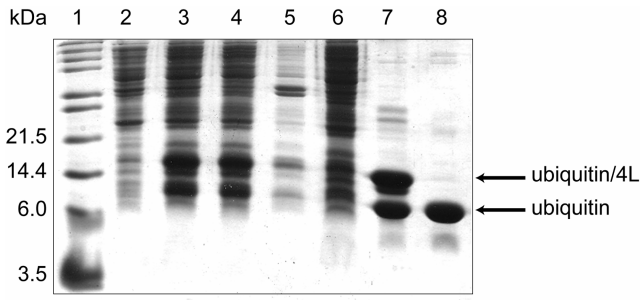

Figure 2. Purification of syndecan 4L produced from 1 liter LB medium as monitored by 16% SDS-PAGE. Lane 1, size marker; lane 2, whole cell lysate before IPTG induction; lanes 3 and 4, supernatant and pellet of cell lysate, respectively; lane 5, flow-through fraction of supernatant from HiTrap Chelating HP column; lane 6, fraction that was bound to the HiTrap column; lane 8, sample of lane 7 after YUH cleavage reaction.

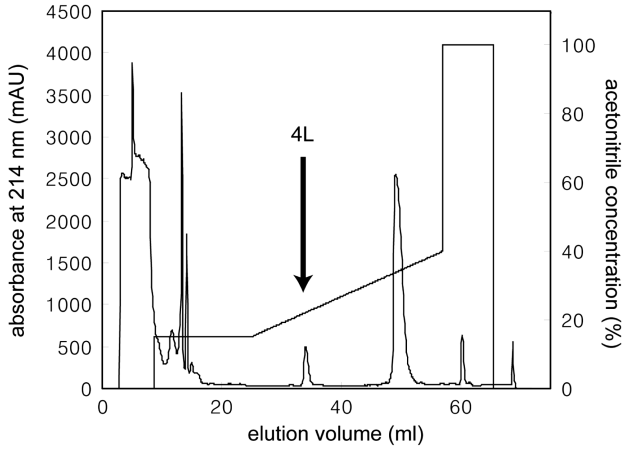

Figure 3. The elution profile from the reverse phase chromatography. The peak corresponding to 4L is marked with an arrow. The concentration of acetonitrile during the gradient elution is also shown.

this amount included not only the ubiquitin with full-sized 4L, but also the ubiquitin molecule alone and that with partial 4L. Thus, it would be safe to say that amount of the ubiquitin with full-sized 4L was half of the calculated mass.

Purification of 4L. The original purification strategy was to exchange buffers from imidazole-rich to an imidazole-free state before cleaving the fusion protein by YUH, which would help remove both of the His-tagged YUH and ubiquitin by filtering them through the HiTrap Chelating HP column. However, not all of the ubiquitin molecules were filtered out, but we always found small amount of contaminating ubiquitin molecules in the flow-through fraction. Since the filtering step did not work satisfactorily, we directly added YUH and β-mercaptoethanol to the eluted fraction from the HiTrap Chelating HP column without changing to the imidazole-free buffer. The prepared YUH was effective in cleaving ubiquitin from 4L. One hour was enough to cleave around 25 mg of the fusion protein with 50 μg of YUH. Following cleavage, the entire reaction mixture was loaded onto a Resource RPC column in 2 mL aliquots (GE Healthcare, Piscataway, NJ, USA). The volume of the cleavage reaction mixture was around 30 mL, so we repeated the reverse phase chromatography step 15 times. The peptide was eluted at around 23% acetonitrile, and all the 4L-containing fractions were pooled and lyophilized (Fig. 3). The final yield of 4L was around 3 or 1.5 mg per liter of LB or minimal medium, respectively. MALDI-TOF was used to verify the purified peptide (Fig. 4). MALDI-TOF showed a major peak at 3449.4 Da, which is in good agreement with the theoretical molecular weight of 3450.9 Da.

HSQC spectrum of 4L. The HSQC spectrum showed 24 well resolved peaks (Fig. 5). Among these peaks the two around (^{15}N, ^{1}H) = (113 ppm, 6.95/7.65 ppm) were characteristic resonances of the sidechain amide protons of Asn or Gln. There is one Asn but no Gln in 4L, so these peaks were tentatively assigned to the sidechain amide protons of Asn-24, and this finding provided another assurance of the purified 4L.

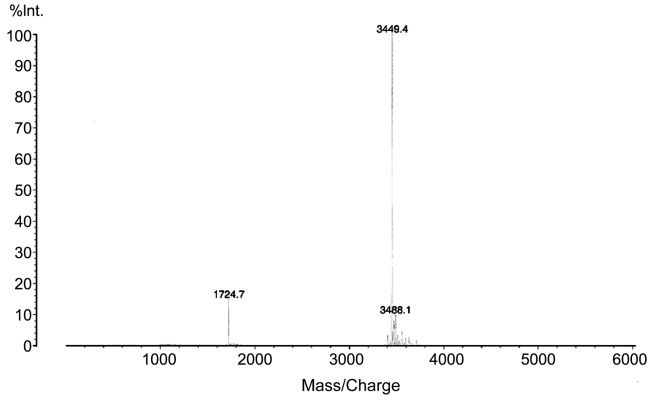

Figure 4. MALDI-TOF spectrum of purified syndecan 4L. Single and double charged molecular ions are seen at m/z = 3448.4 and 1724.4, respectively.

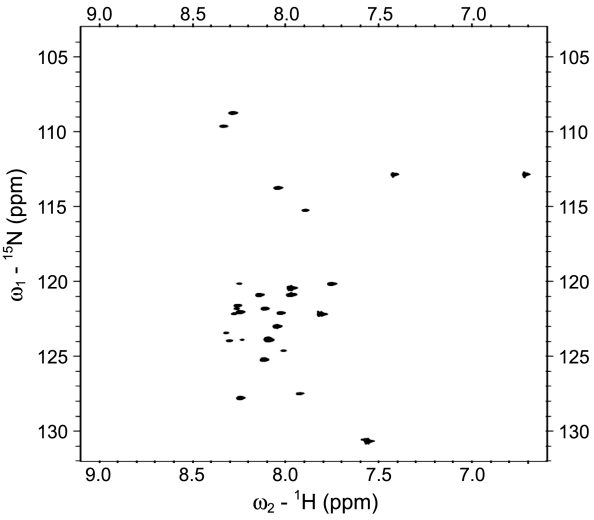

Figure 5. [^{1}H-^{15}N] HSQC spectrum of 4L recorded at 283 K.

Conclusion

The methods in this report present a very efficient production/purification procedure for the target peptide. The ubiquitin fusion system was proven to be successful and as such to offer a competitive alternative compared to other fusion systems such as GST or MBP. This system gets maximally effective when it comes to the recombinant peptide preparation. The biggest advantage of the ubiquitin fusion system over the others stems from the small size of ubiquitin. Because it is small, the net yield of the target peptide is higher for the ubiquitin fusion system compared to the others if the fusion proteins were produced in a similar quantity. The YUH efficiently cleaved the ubiquitin fusion protein even in the high salt condition like the elution buffer of the HiTrap Chelate column, and since it can easily be prepared in the homemade fashion, it provides a cost-effective way of preparing the target peptide as compared to the expensive protease like thrombin. The NMR and X-ray structural studies are now under way.

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