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Optimization of Expression, Purification, and NMR Measurement for Structural Studies of Syndecan-4 Transmembrane Region

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Abstract: Syndecan-4 is a transmembrane heparan sulfate proteoglycan, which is a coreceptor with integrins in cell adhesion. To get better understand the mechanism and function of Syndecan-4, it is critical to elucidate the three-dimensional structure of a single transmembrane spanning region of them. Unfortunately, it is hard to prepare the peptide because syndecan-4 is membrane-bound protein that transverse the lipid bilayer of the cell membrane. Generally, the preparation of transmembrane peptide sample is seriously difficult and time-consuming. In fact, high yield production of transmembrane peptides has been limited by experimental adversities of insufficient yields and low solubility of peptide. Here, we demonstrate experimental processes and results to optimize expression, purification, and NMR measurement condition of Syndecan-4 transmembrane peptide.

Keywords: Syndecan-4, Transmembrane peptide, NMR, HPLC, Purification

INTRODUCTION

Syndecans are transmembrane heparan sulfate proteoglycans (HSPGs) and present on most cells. [1] They are known to link cell to the extracellular domain as well as to function as heparan sulfate receptors in fibroblast growth factor signaling. [2-5] Syndecan-4 is a member of the syndecan family,

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which consists of 4 transmembrane heparan sulfate proteoglycans. Syndecan-4 is known to play an important role in focal adhesion. They bind to various bio-mediators, such as basic fibroblast growth factor, midkine, and tissue factor pathway, via its heparan sulfate chain and regulate cell-matrix interactions. Signaling by focal adhesions has an effect on cell adhesion, apoptosis, and gene expression. [6] Syndecan-4 has an N-terminal signal peptide, an ectodomain, a single hydrophobic transmembrane domain, and a short C-terminal cytoplasmic domain. [7] Although the structure of cytoplasmic domain is already known, the mechanism of signal transduction is not clearly understood yet.

It has been known that most transmembrane protein perform crucial roles in the function of cells. Within the cell membrane, signal transduction is initiated and materials and energy are exchanged. Cell–cell interaction, as well as cell–environment interaction, is especially necessary for the survival. However, researching transmembrane proteins has been restricted by a lot of experimental difficulties. The problem is the relatively low abundance of transmembrane proteins as compared with other cellular proteins, and the purification of them remains quite difficult due to the hinderence by inherent toxicity and aggregative nature of themselves. [8, 9] Recently, we achieved success to produce Syd4-TM peptides and try to examine the structure of the single transmembrane domain of Syndecan-4 (Syd4-TM) in the membrane-like environments. The peptides were overexpressed in the form of fusion protein *in Escherichia coli*, and purified with several chromatography techniques. To get large amounts of Syd4-TM peptides enough to solid-state NMR structural studies, it was

required to design a vector construction suitable for the high-level expression, to screen *E. coli* strain, and to optimize the conditions of expression and purification. And we also optimized NMR measurement conditions for well ordered Syd4-TM peptides in micelle environments. In this paper, we demonstrate the optimization of expression, purification, and NMR measurement condition for structural studies of Syd4-TM peptides.

MATERIALS AND METHODS

Expression

Detail Methods for suitable vector construction and transformation of Syndecan 4 transmembrane region are explained in previous paper. [8] A single colony was inoculated to 50 ml LB media and grown for overnight at 37 °C. The fully grown culture was transferred in 1 L M9 minimal media and then grown at 37 °C. Fused proteins were induced at OD_{600} 0.5~0.6 added from 1mM isopropy-β-D-thiogalactoside (IPTG). After 16 hrs of induction, the cells were harvested by centrifugation and stored at -80 °C. [9, 10, 11, 12]

Purification

The cell pellet was dissolved with lysis buffer containing lysozyme and ultrasonicated in ice bath. The lysate was centrifuged at 13,200 rpm for 30 min at 4 °C, and insoluble fraction was resuspended in a binding buffer containing 6 M Guanidium-HCl. The suspension was re-centrifuged

at 13,200 rpm for 35 min at 4 °C. The clear supernatant after centrifugation was loaded Ni-NTA column, which had been previously charged with $NiSO_4$ and equilibrated with binding buffer containing 6 M Guanidium-HCl. After washing column with 4 volumes of washing buffer, fused protein was eluted with four volumes of elution buffer. To remove guanidine and salts, elutes were dialyzed against deionized water. The white precipitates of fusion protein were collected and lyophilized. [9, 10, 11]

Cleavage

Lyophilized fusion protein was dissolved in 70% formic acid, and cleavage to remove the fusion partner KSI and His_6-tag was initiated by adding cyanogen bromide. Reaction mixture was incubated at room temperature under darkness for 5 hrs. Reaction mixture after cleavage was diluted five-fold with deionized water, and then dialyzed in a 1,000 MWCO membrane to remove the impurities, such as cyanogen bromide and formic acid. Finally, dialyzed solution was lyophilized. [9, 10, 11]

HPLC

Final purification of Syd4-TM peptide was tested at three different conditions using preparative reversed-phase HPLC system (Waters Delta 600, USA). First, protein mixtures containing Syd4-TM were dissolved in 1:4 TFE/MC at a concentration of 2mg/ml, and then centrifuged at 15,000 rpm for 1 hour at 4 °C. Clear supernatant was filtered using 0.45 μm syringe filter, and was then injected to

HPLC equipped with a Delta Pak C4 column (7.8 × 300 mm, Waters). Peptides were eluted with a linear gradient of ACN in water containing 0.1 % TFA. Second, protein mixtures were dissolved in 1:4 HFIP/MC at a concentration of 2mg/ml, and then centrifuged at 15,000 rpm for 1 hour at 4 °C. Clear supernatant was filtered using syringe filter, and was then applied to a Delta Pak C18 column (7.8 × 300 mm, Waters). Peptides were eluted with a linear gradient of ACN in water containing 0.1 % TFA. Third, protein mixtures were dissolved in 1:4 HFIP/MC at a concentration of 2mg/ml, and then centrifuged at 15,000 rpm for 1 hour at 4 °C. Clear supernatant was filtered using 0.45 μm syringe filter also, and was then applied to a Delta Pak C18 column (7.8 × 300 mm, Waters). Peptides were eluted with a linear gradient of eluent C (95% water with 0.1% TFA+3% isopropanol+2% acetonitrile) and D (5% water with 0.1% TFA+ 47% isopropanol,+28% acetonitrile+20% TFE). HPLC separation was monitored at 220 nm and 280 nm using a PDA detector at a flow rate of 3 ml/min. Each fraction was pooled and identified by 12 % tris-tricine polyacrylamide gel electrophoresis (PAGE). The resultant of Syd4-TM fractions was collected and lyophilized. The molecular weight and the purity were characterized by mass spectrometry also.

Solution NMR spectroscopy

Micelle Samples were prepared by dissolving 1 mg of ^{15}N-labeled Syd4-TM peptides in 300 μL of 150 mM DPC-d_{38} containing optimized NMR buffer containing 10 mM Na_2HPO_4, 1 mM NaN_3, 40 mM DTT, 90 % H_2O and 10 % D_2O). 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 298 to 318 K in 5 degrees increments, and also changing pH values of 4.0, 4.5, 5.0, and 5.5. The two-dimensional $^{1}H/^{15}N$ 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 with 8–16 scans each in t1 and 1024 data points in t2 time domains, and data processing was performed using TOPSPIN 2.1 software (Bruker Biospin).

RESULTS AND DISCUSSION

Expression and purification

High-level expression of KSI-Syd4_TM-His_6 (18 kDa) was induced by addition of 1 mM IPTG at OD_{600} 0.5 (Fig. 1, lane 2). Peptide purity and identities at each purification steps were checked by 12 % tris-tricine PAGE and 14 % SDS PAGE, as shown in Fig. 1. Expressed fused protein was easily found in 14 % SDS PAGE rather than 12 % tris-tricine PAGE. On the other hand, Identification of Syd4-TM peptide after CNBr cleavage or HPLC purification was better in 12 % tris-tricine PAGE than in 14 % SDS PAGE. Since KSI-fused proteins were found in inclusion body (Fig. 1, lane 4) 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. From the result of SDS PAGE under reducing condition, we found that purified fusion protein formed as a dimer (Fig. 1, lane 5). The final yield of purified fusion protein was about 120 mg per liter culture.

Cleavage

KSI fused protein was chemically cleaved with CNBr to release the Syd4-TM peptide (Fig. 1, lane 6). The reaction mixture was diluted five-fold with deionized water, and then dialyzed overnight at room temperature against 5 L of deionized water in 1,000 MWCO dialysis bags. Subsequently, the dialyzed mixture was lyophilized.

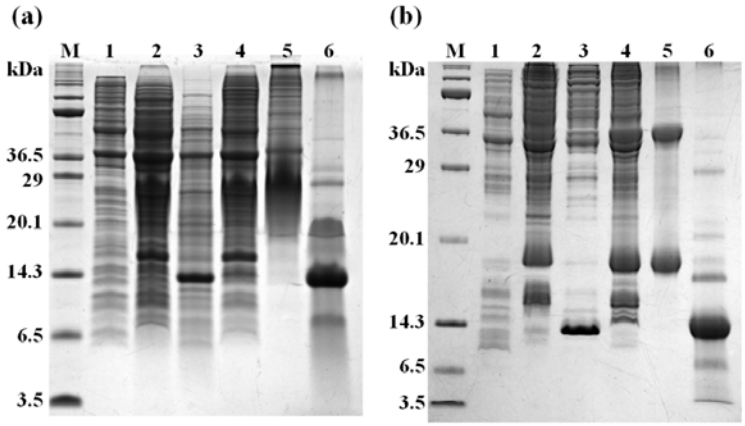

Figure 1. Coomassie blue stained (a) 12% tris-tricine PAGE and (b) 14% SDS-PAGE showing samples from expression and Ni-NTA column purification of the KSI fusion protein. Lane 1, cells before IPTG induction; Lane 2, cells after IPTG induction; Lane 3, soluble fraction after lysis; Lane 4, insoluble fraction after lysis; Lane 5, KSI-fused protein purified from a Ni–NTA affinity chromatography; Lane 6, reaction mixtures after CNBr cleavage.

HPLC

Final purification of Syd4-TM peptide was conducted under three different conditions, as described above. Fig. 2(a) shows a reversed phase HPLC chromatogram in the first condition (1:4 TFE/MC, C4 column, eluent A: 95 % ddH_2O, 5% ACN, 0.1% TFA, eluent B: 5 % ddH_2O, 95 % ACN, 0.1 % TFA). From the result of Fig. 2(b), it was not successful because of poor separation. Fig. 3(a) shows a reversed phase HPLC chromatogram in the second condition (1:4 HFIP/MC, C18 column, eluent A, B). From the result of Fig. 3(b), separation under the second condition was a little better than the first condition, but it was still not good enough to purify Syd4-TM peptide. Finally, we changed the mobile phase compositions; eluent C: 95 % water, 3 % isopropanol, 2 % acetonitrile, 0.1 % TFA, eluent D: 5 % water, 47 % isopropanol, 28 % acetonitrile, 20 % TFE, 0.1 % TFA. Fig. 4(a) shows a reversed phase HPLC chromatogram in the third condition (1:4 HFIP/MC, C18 column, eluent C, D). From the result of Fig. 4(b), KSI fusion partner (~14 kDa) was eluted at 57 min (lane 1), and Syd4-TM peptide was eluted at 75-85 min (lane 2, 3) with two peaks from different forms of

oxidized homoserine and homoserine lactone. Purified Syd4-TM peptide also formed as a dimer under reducing condition (Fig. 4(b), lane 3). Finally, we obtained high purity peptides for uniformly and selectively ^{15}N labeled Syd4-TM peptide with 4-5mg/L in M9 minimal medium. The molecular weight and the purity of the isolated peptides were characterized by MALDI mass spectrometry. The measured molecular mass matched well with theoretical molecular weight of Syd4-TM (data not shown)

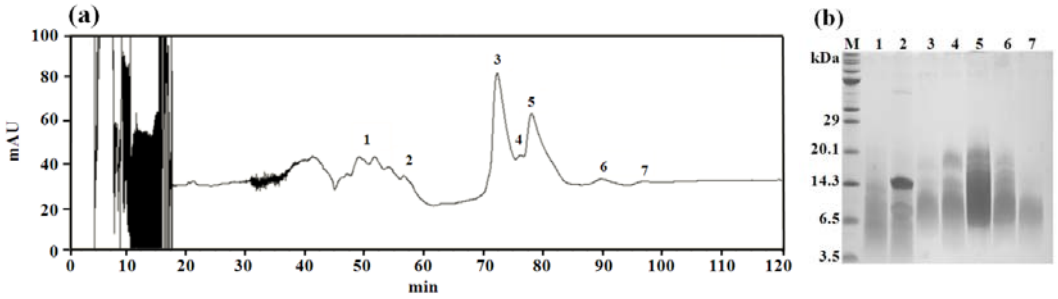

Figure 2. (a) Reversed phase HPLC chromatogram under the first condition. Cleavage reaction mixtures containing Syd4-TM were dissolved in 1:4 TFE/MC at a concentration of 2mg/ml. Peptides were eluted with a linear gradient of ACN in water containing 0.1 % TFA on the C4 column. Eluent A: 95 % ddH_2O with 0.1% TFA and 5% ACN, Eluent B: 5 % ddH2O with 0.1% TFA and 95 % ACN. (b) 12 % tris-tricine PAGE diagrams for HPLC fractions.

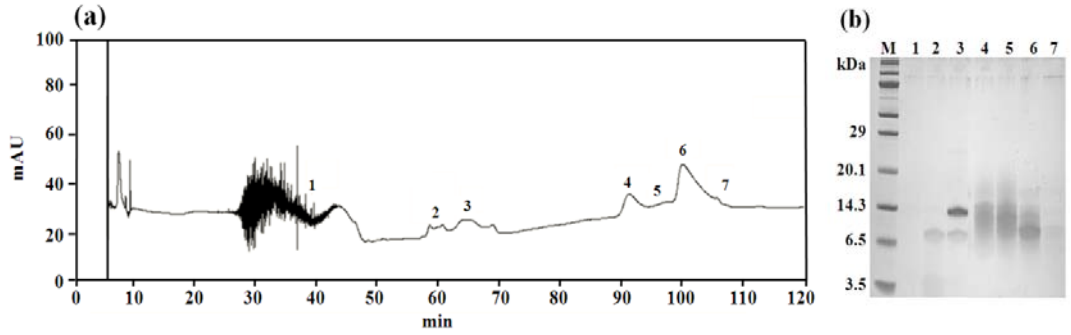

Figure 3. (a) Reversed phase HPLC chromatogram under the 2nd condition. Cleavage reaction mixtures containing Syd4-TM were dissolved in 1:4 HFIP/MC at a concentration of 2mg/ml. Peptides were eluted with a linear gradient of ACN in water containing 0.1 % TFA on the C18 column. Eluent A: 95 % ddH_2O with 0.1% TFA and 5% ACN, Eluent B: 5 % ddH2O with 0.1% TFA and 95 % ACN. (b) 12 % tris-tricine PAGE diagrams for HPLC fractions.

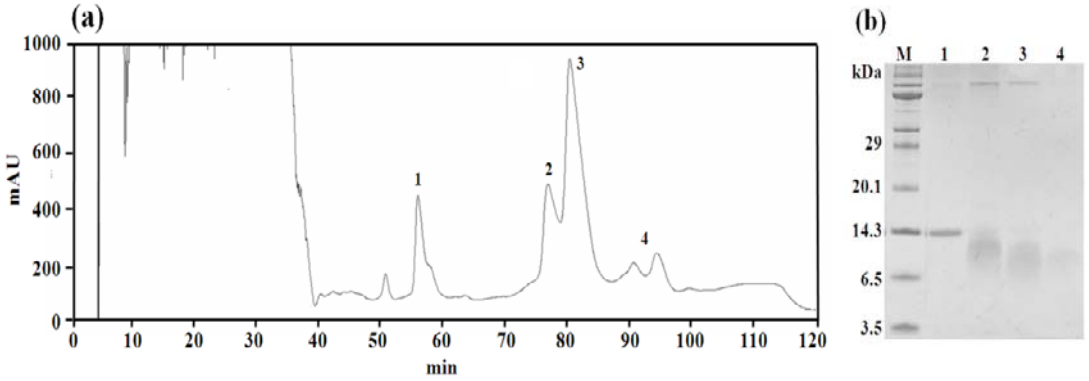

Figure 4. (a) Reversed phase HPLC chromatogram under the third condition. Cleavage reaction mixtures containing Syd4-TM were dissolved in 1:4 HFIP/MC at a concentration of 2mg/ml. Peptides were eluted with a linear gradient of eluent C and D on the C18 column. Eluent C: 95 % water containing 0.1 % TFA and 3 % isopropanol and 2 % acetonitrile , Eluent D: 5 % water

containing 0.1 % TFA and 47 % isopropanol and 28 % acetonitrile and 20 % TFE (b) 12 % tris-tricine PAGE diagrams for HPLC fractions.

Solution NMR spectroscopy

Purified ^{15}N-labeled Syd4-TM peptides were characterized 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. Fig. 5 shows ^{1}H/^{15}N 2D HSQC spectra of Syd4-TM measured at pH 4.0, 4.5, 5.0, and 5.5. Spectral resolutions at different pH conditions were quite similar, but as increasing pH values, some resonances disappeared. Temperature was also optimized by acquiring ^{1}H/^{15}N 2D HSQC spectrum from 298 to 318 K in 5 degrees increments, as shown in Figure 6. The spectral patterns at different temperature were also quite similar. Among them, the spectral resolution was the best at 313 K. 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.

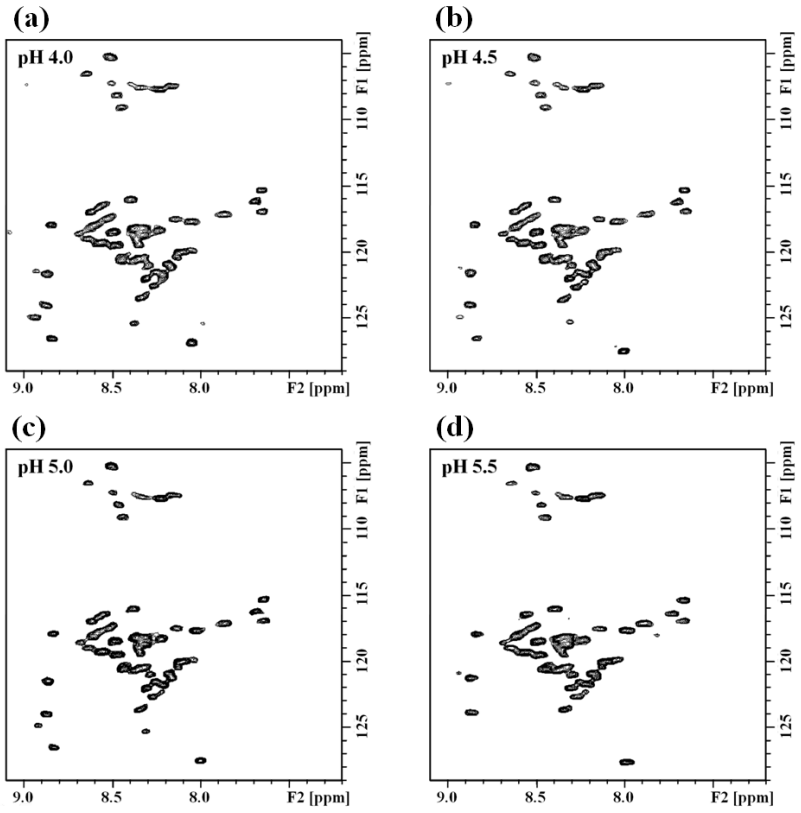

Figure 5. 1H/15N 2D HSQC spectra of Syd4-TM measured at (a) pH 4.0, (b) pH 4.5, (c) pH 5.0, and (d) pH 5.5.

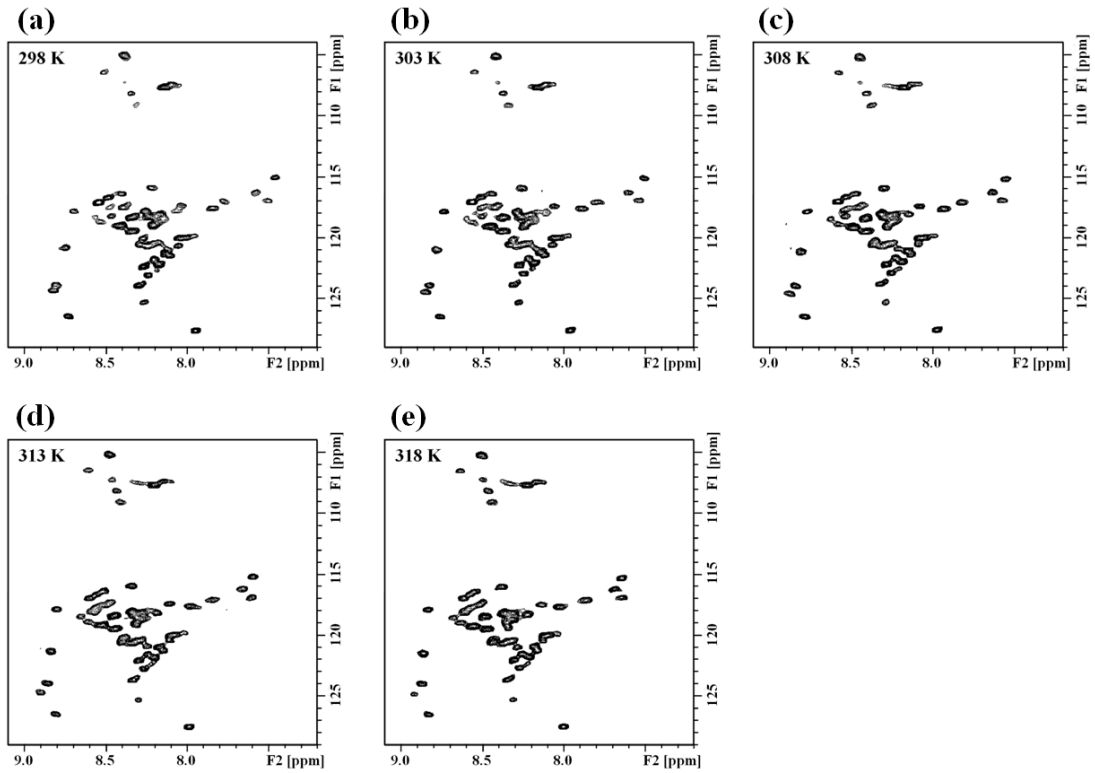

Figure 6. 1H/15N 2D HSQC spectra of Syd4-TM measured at (A) 298 K, (B) 303 K, (C) 308 K, (D) 313 K, and (E) 318 K

CONCLUSION

In this paper, we demonstrate the optimization of expression, purification, and NMR measurement condition for structural studies of Syd4-TM peptides. Milligram quantities of Syd4-TM peptides were obtained by using preparative reversed-phase HPLC. Purified Syd4-TM peptides

formed as a dimer under reducing condition. The number of resonances in a $^{1}H/^{15}N$ 2D HSQC spectrum was almost matched to the number of amino acid of dimeric Syd4-TM peptides. The methods and conditions used in this study will be helpful to produce other transmembrane peptides and to perform the structural studies of micelle samples by solution NMR spectroscopy.

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REFERENCES

1. A. C. Rapraeger, *J Cell Biol.* **149**, 995. (2000).
2. K. Elenius, and M. Jalkanen, *J. Cell Sci.* **107**, 2975. (1994).
3 M. Salmivirta, and M. Jalkanen, *Birkhauser Verlag.* **51**, 863. (1995).
4. A. Woods, *J. Clin. Invest.* **107**, 935. (2001).
5. S. M. Kim, *J. Kor. Magn. Reson.* **13**, 1. (2009).
6. A. Woods, and J. R. Couchman, *Curr. Opin. Cell Biol.***13**, 578. (2001).
7. M. Bernfield, R. Kokenyesi, M. Kato, M. T. Hinkes, J. Spring, R. L. Gallo, and E. J., *Annu. Rev. Cell. Biol.* **8,** 365. (1992).
8. T. K. Park, M. H. Lee, and Y. Kim, *Proc. Biochem.* **46**, 1161. (2011).
9. T. J. Park, S. S. Choi, G. A. Gang, and Y. Kim, *Protein Expr. Purif.* **62**, 139. (2008).

10. T. J. Park, J. S. Kim, and Y. Kim, *Protein Expr. Purif.* **65**, 23. (2009).
11. T. J. Park, S. Y. Im, J. S. Kim, and Y. Kim, *Proc. Biochem.* **45**, 682. (2010).
12. J. S. Kim, T. J. Park, and Y. Kim, *J. Kor. Magn. Reson.* **13**, 96. (2009).