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Optimized purification and characterization of expressed hMC4R-TM2

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Abstract: Human melanocortin-4 receptor (hMC4R) among MC-Rs, expressed in the brain, is in charge of the control on energy homeostasis and food intake. The structure and function of human MC4R have been studied to understand their essential function and roles. To investigate the structure and function, it is necessary to prepare sufficient amounts of proteins. However, their expression and purification is demanding and time-consuming due to their innate insoluble and toxic properties. The heterozygous mutations of hMC4R, exchange of Asp 90 to Asn located in second transmembrane, cause severe obesity in human. To obtain purified hMC4R wt-TM2 for structural studies, it was first over-expressed and purified by fast protein liquid chromatography (FPLC) and then solution NMR studies were performed to get high-resolution spectra. In here, we established optimized purification scheme to get more purified target peptide.

Keyword: MC4R, transmembrane, NMR, FPLC, Purification, CNBr cleavage

INTRODUCTION

The melanocortin receptors (MC-Rs) that contain five subtypes are member of the G protein-coupled receptor (GPCR).[1] The MC-Rs are responsible for the control of energy expenditure and intake.[2] Melanocortins exert various functions by activating MC-Rs of cell surface integral membrane protein. Five subtypes in melanocortin receptors have been cloned and are named from

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MC1R to MC5R in accordance with the sequence of their cloning. They reveal dissimilar functions based on localization. The MC1R is the classical melanocyte-stimulating hormone receptor (MSHR) expressed in the mammalian skin and hair color that controls pigment.[10] The MC2R is the classical adrenocorticotropic hormone receptor (ACTHR) that is expressed in the adrenal cortex regulating growth and adrenal steroidogenesis.[11] The MC3R and MC4R are called neural MC-Rs because of expression in the brain.[12] The MC5R is induced in their exocrine glands and regulates the serum of these glands.[3,4] All melanocortin receptors are signaled principally through intracellular cyclic adenosine monophosphate.[5] The MC4R of melanocortin receptors, expressed in the brain, plays a significant role in the control of energy homeostasis and food intake[6] , because hypothalamic melanocortin system consisting of the MC4R and its ligands maintains these functions as known in numerous studies.[7] The mutations of MC4R, exchange of Asp90 to Asn located in second transmembrane domain, cause an early-onset form of obesity in human. Functional analysis of this variation indicates that obesity-associated defects ranging from constitutive activation to loss of function appear.[8] Single missense mutation (D90 to N) in MC4R gene decreases MC4R signaling and cAMP content.[6]

In order to study the function and structure of hMC4R wt -TM2 peptide, we prepare suitable amounts of protein before, even though, expression and purification of transmembrane protein is severely difficult, time-consuming, and a lot of cost because of their insolubility and toxicity**.** [13, 14]However, we could find that target peptide contains decent amount of KSI-fragment. Therefore, we

spend more time optimizing the purification method such as chemical cleavage, FPLC, and dialysis.

EXPERIMENTAL METHODS

Expression of the KSI- hMC4R TM2 fusion protein

For the expression, single colony of fusion protein was used for cell culture dish containing LB medium with 13.2 μM carbenicillin (Amresco, USA). This colony was cultured overnight at 37 ℃. Pre-culture was incubated from cell stock in 50 ml LB medium that contains antibiotics to prevent other bacteria from growing in shaking incubator at 37 ℃ for 16 hours. After finished pre-culture, 10 ml of this LB solution was transferred into 1 L of M9 minimal media containing 1 g of ^{15}N-enriched ammonium sulfate (Cambridge Isotope Lab, USA), and the culture was then grown in shaking incubator at 37 ℃. When the optical density at 600 nm attained 0.5, 1 mM IPTG (Noble Biosciences, Korea) was added into the cell culture for induction of fusion protein. The cells were incubated 16 hours after induction. The cells were harvested by centrifuge with 6000 rpm at 4 ℃ for 30 min and were placed in -80 ℃ refrigerator over 3 hours.

Purification of the KSI- hMC4R TM2 fusion protein

The frozen pellet harvested was lysed from 100 ml lysis buffer (15% glycerol, 20 mM Tris, 500 mM NaCl) and 0.05 g lysozyme (Sigma, USA) for about 3 hours. After cell lysis, it was lysed by mechanical method with ultra-sonication operated four cycles at 2.5 s on and 9.9 s off for 19 min on ice bath. The cells were disrupted chemically and mechanically, and then were centrifuged with 13,200 rpm at 4 ℃ for 30 min. The pellet containing fusion protein was dissolved in binding buffer (6 M guanidine-HCl, 20 mM Tris, 5 mM Imidazole, 500 mM NaCl, pH 8.0) for overnight to denature its folded structure. Subsequently, to remove impurities it was necessary to centrifuge mixture at with 13,200 rpm 4 ℃ for 30min. Ni-NTA column was used for separation of fusion protein after centrifuge. The fusion protein was eluted with elution buffer (500 mM NaCl, 10 mM Tris, 6 M guanidine-HCl and 500 mM Imidazole, pH 8.0). To remove salt and denaturing agent, the eluate was dialyzed against deionized distilled water using a 10 kDa molecular weight cutoff (MWCO) tubing for a day at room temperature. The fusion protein after dialysis was refolded to their native insoluble state and formed as white precipitates. This precipitates were collected and lyophilized.

The fusion protein after lyophilization was dissolved in 70% formic acid (Sigma, USA) at a concentration of 5 mg per milliliter and fresh solid state cyanogen bromide (Sigma, USA) was added at a concentration of 100 mg per milliliter to cleave chemically the methionine residues from the fusion protein. And then, the mixture was placed in the dark room at room temperature for 5 hours. Because 5 hours of cleavage induces unnecessary KSI fragment from the fusion protein, we selected different cleavage time of 30 mins, 1 hour and 5 hours for comparison of KSI fragment. After cleavage, it was dialyzed against deionized distilled water using 1000 MWCO tubing for a day at

room temperature to remove impurities containing cyanogen bromide and formic acid. The mixture was collected and lyophilized. The purity of protein at each step was confirmed by 12% Tris-Tricine PAGE.

Purification of the hMC4R TM2 peptides

For purification of the cleaved fusion protein, fluffy products were dissolved in FPLC buffer (100 mM Na_2HPO_4, 20 mM DTT, 4 mM SDS, 1 mM EDTA, 1 mM NaN_3) with bath sonication and filtered off impurities using a 0.45 μm pore size syringe filter. The solution was applied to a Superdex 200 prep grade column on AKTA FPLC system (GE Healthcare, USA). The column was eluted with FPLC buffer at room temperature and a flow rate of 1 ml/min. The sample fractions were collected by auto-fraction. Each fraction was confirmed by 12% Tris-Tricine PAGE. Eluate including micellized hMC4R TM2 peptides was dialyzed against deionized distilled water using 1000 MWCO tubing for four days at room temperature for removal of SDS and salts and followed by lyophilization. Dialysis time is also optimized.

Identification of the hMC4R TM2 peptides

In order to identify purified hMC4R TM2 peptides, these peptides were analyzed by AB SCIEX 4800 matrix-assisted laser desorption ionization time of flight / time of flight mass

spectrometry (MALDI/TOF MS). The peptides were dissolved in mass buffer (50% ACN/50% H_2O, 0.1% TFA) with bath sonication. A 6 ml of peptide solution and 6.5 ml of matrix, a-cyano-4-hydroxycinnamic acid (CHCA) for detection of a low mass-to-charge ratio, were mixed in eppendorf tube. A 1 ㎕ of this mixture was deposited on mass plate and dried for crystallization. And then, the mass plate was loaded into mass analyzer. To improve the resolution, this experiment was conducted on reflector mode. The laser used for analysis was diode-pumped Nd:YAG at 355 nm and pulse rate was 200 Hz. Also, circular dichroism (CD) was conducted to analyze roughly a secondary structure of this peptide. CD experiment was performed by using a Jasco J815 spectropolarimeter with 1 mm path length cell. 0.1 mM of the hMC4R TM2 was dissolved in sterilized water, CD salts (10 mM Na_2HPO_4) and SDS. For observation of the dependence on concentration of SDS, samples was made with different concentration of 0 mM, 20 mM, 40 mM, 60 mM, 80 mM, and 100 mM. A pH of each sample was adjusted at 4 and final volume was 400 ㎕. The CD spectrum was recorded from 190 nm to 250 nm with sensitivity of 20 mdeg, step resolution of 0.2 nm, scan speed of 50 nm/min, accumulation of 5 and response of 0.25 s. Finally, to assign amino acid sequence of the hMC4R-TM2 peptide, solution NMR experiment was carried out using a Bruker AVANCE 800 MHz spectrometer in KBSI. The uniformly ^{15}N-labeled hMC4R-TM2 peptides were solubilized in a 500 mM SDS, 40 mM DTT, 1 mM NaN_3, 10 mM Na_2HPO_4, 90% H_2O and 10% D_2O at pH 4.0, so that final volume was 300 ㎕ and final concentration was 0.5 mM. 2D ^1H-^{15}N heteronuclear single quantum coherence (HSQC) spectrum was acquired at 313-323 K with 128 increment (64-8 scans each) in t1 and 2048 data points in t2. Data processing was carried out on TOPSPIN 2.1 software.

RESULTS AND DISCUSSION

Expression of the KSI- hMC4R TM2 fusion protein

High-level expression of the KSI-hMC4R TM2 peptides was induced in LB media by addition of 1 mM IPTG at OD_{600} of 0.5, as shown in Fig 1. The value of OD_{600} was maximized 16 hours after induction. The grown cells were collected and harvested by centrifugation. And then, it was frozen for 3 hours to lyse cells easily. The frozen cells were lysed by lysis buffer and lysozyme about 3 hours and broken by ultra-sonication. After centrifugation, insoluble fusion protein was found in pellet part of cell lysate as shown in Fig. 1, lane 5 and it was not in supernatant as shown in Fig. 1, lane 4. The pellet was dissolved by binding buffer and denatured with 6M guanidine-HCl. Recombinant protein tagged with 6 Histidine residues was efficiently purified by Ni-NTA column and then refolded by dialysis. The purified fusion protein was obtained by lyophilization as shown in Fig. 1 , lane 6. Its yield was 200-210 mg per liter of M9 media.

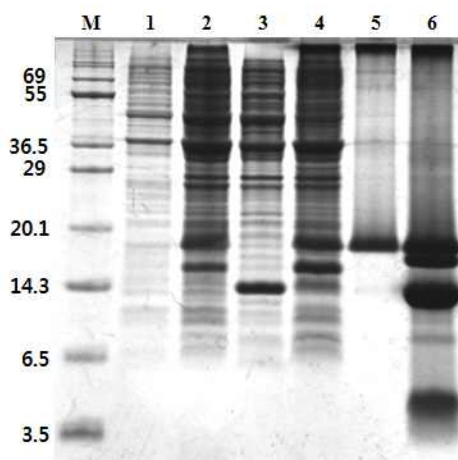

Figure 1. 12% Tris-Tricine PAGE diagram to identify purification step of expression and purification for hMC4R-TM2. The fusion protein was cleaved at methionine residues under 70% formic acid for 5 hours. M; protein-blue molecular weight maker, Lane 1; the cells before induction, Lane 2; the cells after induction , Lane 3; the supernatant part after cell lysis, Lane 4; the pellet part after cell lysis with a fusion protein band at 17.9 kDa, Lane 5; Eluted fusion protein with elution buffer, and Lane 6; Cleaved fusion protein mixtures.

Purification of the KSI- hMC4R TM2 fusion protein

The fusion protein was dissolved in 70% formic acid and chemically cleaved with CNBr. The band intensity of fusion protein, 17.9 kDa, was reduced and new four bands corresponding to the KSI of fusion partner protein (13.5 kDa), hMC4R-TM2 (3.3 kDa), and incompletely cleaved fusion proteins (16.8 kDa, 4.6 kDa) appeared as shown in Fig. 1, line7. To compare band intensity of hMC4R-TM2 depending on cleavage time, 12% Bis-Tris PAGE was conducted as shown in Fig. 2. The band intensity of peptide was increased by increment of cleavage time. And band intensity of KSI fragment was also increased at the same time, so optimization of cleavage time was needed. The

gel filtration chromatography on FPLC system was chosen as final purification method for hMC4R-TM2 peptide because of its hydrophobicity. The CNBr cleavage mixtures were dissolved in a FPLC buffer with bath sonication and injected through super-loop into Superdex 200 prep grade column equilibrated with FPLC buffer. Its chromatogram obtained from the UV detector at 280 nm is shown in Fig. 3. But, hMC4R-TM2 peptide could be not detected in the chromatogram at 280 nm because it doesn't have tyrosine and tryptophan residues that have aromatic side chains. So, each fraction from FPLC system was analyzed by 12% Tris-Tricine PAGE in inset of Fig. 3. Fractions corresponding to the hMC4R-TM2 peptides were gathered and dialyzed to remove salts, SDS, and other residual impurities. After dialysis, this sediment was lyophilized. The final yield of purified hMC4R-TM2 peptides was 2-3 mg per 1 liter of M9 culture.

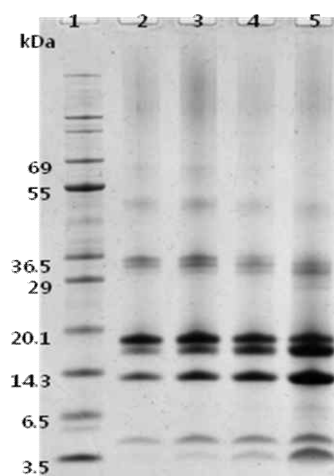

Figure 2. 12% Bis-Tris PAGE diagram corresponding to cleavage time. Lane 1; protein-blue molecular weight maker, Lane 2; 20 mins of cleavage time , Lane 3; 30 mins of cleavage time, Lane 4; 1 hour of cleavage time, and Lane 5; 2 hours of cleavage time.

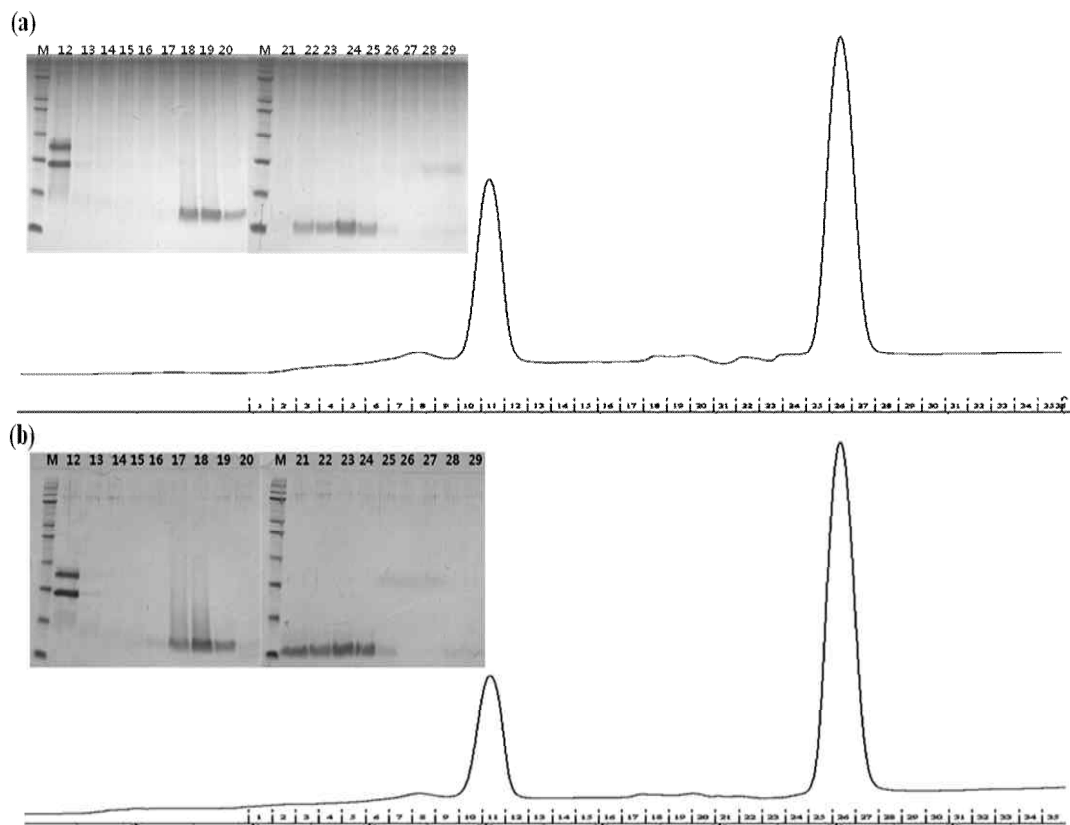

Figure 3. FPLC chromatogram of CNBr cleaved hMC4R-TM2 peptides from the fusion partner. The flow rate was 1 ml/min and detected at 280 nm. (a) fraction numbers from 12 to 29 were displayed by 12% Tris-Tricine PAGE with 5 hours of cleavage time. The fraction number 18-20 display KSI fragment and 22-26 contain hMC4R-TM2 mixtures. (b) fraction numbers from 12 to 29 were displayed by 12% Tris-Tricine PAGE with 20 mins of cleavage time. The fraction numbers 17-19 display KSI fragment and 21-25 contain hMC4R-TM2 mixtures.

Identification of the hMC4R-TM2 peptides

The purified peptide using MALDI TOF/TOF mass spectroscopy has been analyzed prior to the structural studies. The experiment was carried out on reflector mode for improvement of the

resolution. Fig. 4 is shown MALDI-TOF MS spectra of fraction number 24 and 19 in Fig. 3(a) in FPLC chromatogram of peptides cleaved for 5 hours. The uniformly ^{15}N-labeled hMC4R-TM2 peptide gave two mass peaks at 3339.91 m/z and 6679.81 m/z as shown in Fig. 4(a). The peak at 3339.91 m/z is well matched with theoretical mass of uniformly ^{15}N-labeled peptide with free acid form at C-terminus.

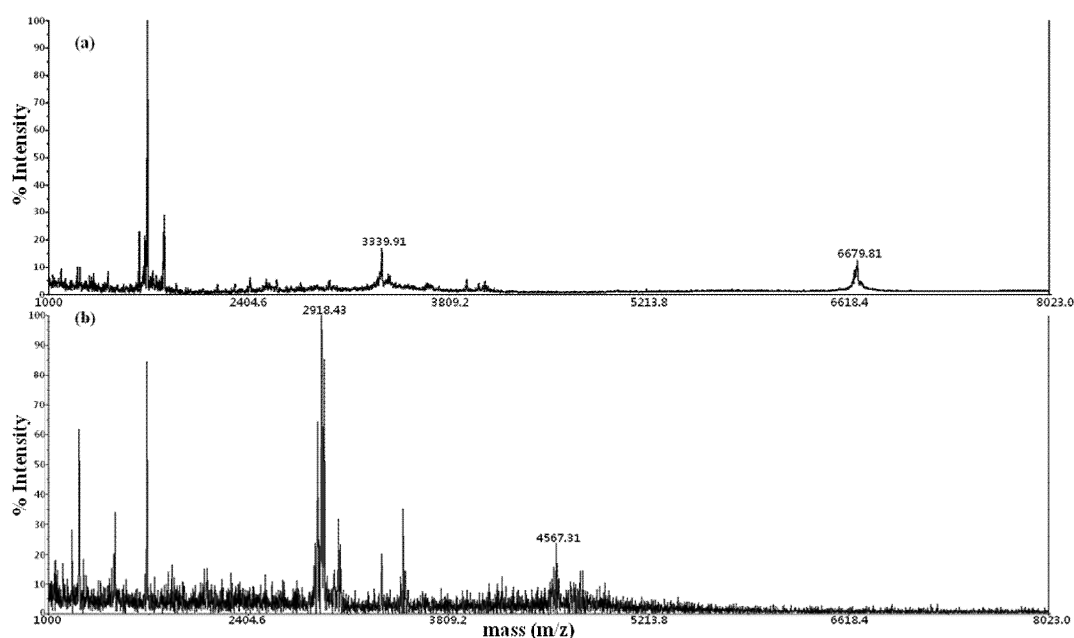

Figure 4. MALDI-TOF MS spectra of (a) the fraction number 24 and (b) the fraction number 19 in Figure 3(a). The peak of 3339.91 m/z in (a) is uniformly ^{15}N-labeled peptide with free acid form. And the peak of 6679.81 in (a) represents dimer of peptide. The peaks of 2918.43 m/z and 4567.31 m/z in (b) represent KSI fragments. These spectra were carried out on a linear mode to observe higher molecular mass ion in order to identify dimer of peptide.

Fig. 5 is shown MALDI-TOF MS spectra of unlabeled hMC4R-TM2 peptide with 1 hour, 2 hours, and 4 hours of chemical cleavage time. The base peak at 3310 m/z corresponding to molecular weight of target peptide is shown in the three spectra. However, impurity peaks at low molecular weight regions near 1656.00 m/z are increased with longer cleavage time.

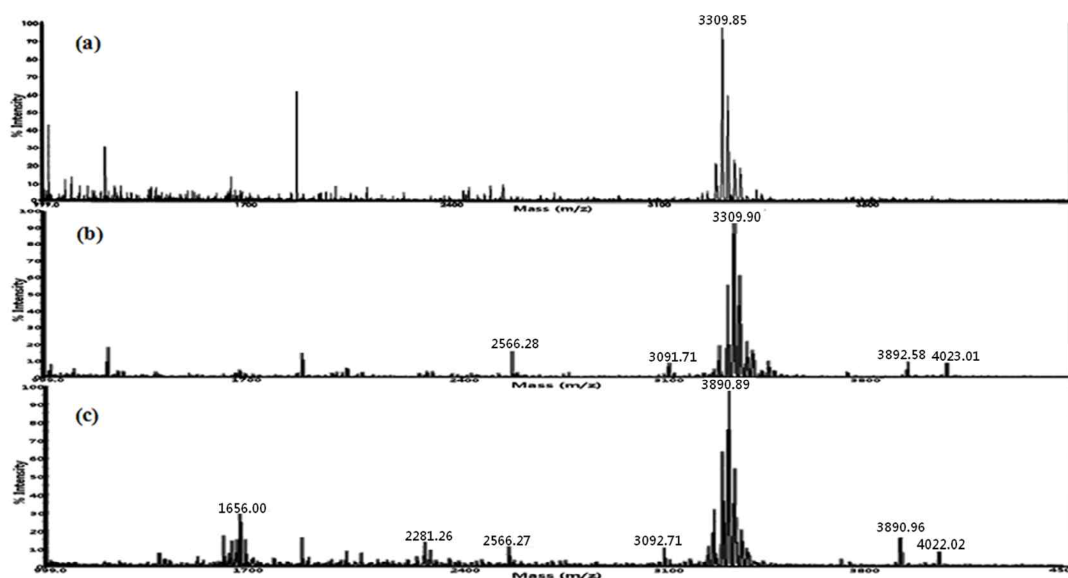

Figure 5. MALDI-TOF MS spectra of unlabeled hMC4R-TM2 with (a) 1 hour, (b) 2 hours, and (c) 5 hours of cleavage time. The peak around 3309.90 m/z is represented by an unlabeled target peptide with free acid form. The impurity such as KSI fragments was increased depending on its cleavage time. These spectra were recorded on reflector mode to obtain high resolution.

In order to analyze a secondary structure of hMC4R-TM2 peptide, CD experiment was performed. The spectrum was recorded from 190 nm to 250 nm. The CD spectrum showed that the

peptide adopts α-helical structure with evidence from the maximum absorption of 190 nm and the minimum absorption observed at both 208 nm and 220 nm as shown in Fig. 6. But CD spectrum at a 0 mM of SDS was displayed random coil structure of peptide. Also this spectrum represented random coil structure independent on its SDS concentration for 4 hours of cleavage time as shown in Fig. 6(d).

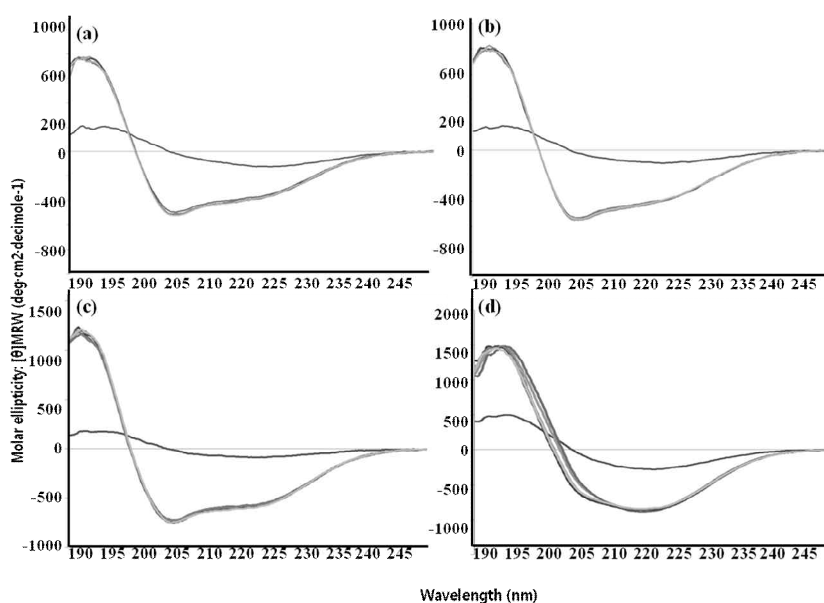

Figure 6. CD spectra of unlabeled hMC4R-TM2 with (a) 20 min, (b) 30 min, (c) 1 hour, and (d) 5 hours of cleavage time. The spectra were recorded from 190 nm to 250 nm.

The structure of hMC4R-TM2 in SDS micelle was analyzed by solution NMR spectroscopy. The ^{1}H-^{15}N HSQC spectra of uniformly ^{15}N-labeled hMC4R-TM2 for 20 min and 5 hours of cleavage time were presented in Fig. 7. The hMC4R-TM2 has 31-mer amino acid. The number of peaks in ^{1}H-^{15}N HSQC spectrum for 20 min of cleavage time accorded with the number of amino acids of

hMC4R-TM2. But the spectrum for 5 hours of cleavage time contains many impurities like KSI fragments.

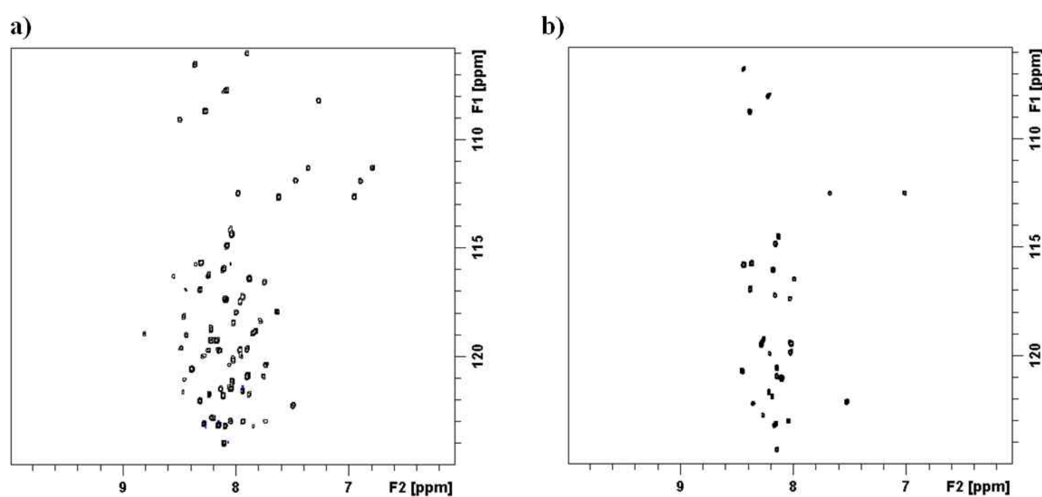

Figure 7. (a) ^{1}H-^{15}N 2D HSQC spectrum of uniformly ^{15}N-labeled hMC4R-TM2 after cleavage during 5 hours in 500mM SDS micelles recorded at 313 K with pH 4.0. (b) ^{1}H-^{15}N 2D HSQC spectrum of uniformly ^{15}N-labeled hMC4R-TM2 after cleavage during 20 mins in 500mM SDS micelles recorded at 323 K with pH 4.0.

Conclusions

Over-expression and optimized purification of hMC4R-TM2 peptide had been performed to achieve enough amount of peptide.[13] There were, however, other impurities except target protein during identification. Therefore, additional purification procedure like cleavage time, dialysis, and

FPLC had to perform for optimization. Tris-tricine PAGE, FPLC chromatography, MALDI-TOF MS, CD, and solution NMR spectroscopy are used to identify the target peptide and purity.

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REFERENCES

1. P. Prusis, P. A. Frändberg, R. Muceniece, I. Kalvinsh, J. E. S. Wikberg, *Biochem. Biophy. Res. Com.* **210**, 205 (1995).
2. N. M. Brito, A. N. Brito, D. J. Baro, C. K. Song, and T. J. Bartness, *Endocrinology*. **11**, 5339 (2007).
3. H Wessells1, N Levine1, ME Hadley, R Dorr, V Hruby, *Int. J. Imp. Res.* **12**, S74 (2000).
4. Y.-X. Tao, *Mol, Cell, Endo* **239**, 1 (2005).
5. Y. Yang, *J. Pharm,* **660**, 125 (2011).
6. K.-S. Kima, J.M. Reecy, W.H. Hsu, L.L. Anderson, M.F. Rothschild, *Dom. Ani. Endo*, **26**. 75 (2004).
7. D. L. Marks, V. Hruby, G. Brookhart, R. D. Cone, *Peptides,* **27**, 259 (2006).
8. C. Vaisse, K. Clement, E. Durand, S. Hercberg, B. Guy-Grand, P. Froguel, *J. Clin. Invest.* **106**, 253 (2000).
9. van der Kraan M . *Endocrinology* **139**, 2348 (1998).
10. K.G.Mountjoy, L.S. Robbins, M.T. Mortrud, R.D.Cone *Science*. **257**, 1248 (1992).
11. V .Chhajlani, J.E.S.Wikberg. *FEBS Lett.* **309**, 417 (1992).
12. I . Gantz et, *J Biol Chem.* **268**, 8246 (1993).
13. T.J. Park, S.S. Choi, A.A. Gang, Y. Kim, *Prot. Exp. Puri.* **62**, 139 (2008).
14. C. Diefenderfer, J. Lee, S. Mlyanarski, Y. G., Kerney J. Glover *Analytical Biochemistry* **384**, 274 (2009).