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Purification and Structural Studies on Human Pro-ghrelin

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Abstract : Ghrelin is a unique peptide hormone that releases growth factor and it stimulates appetite. It comes from pre pro-ghrelin by the post translational modification process and its innate functions are known as food up-take and the growth hormone regulation. Therefore, the structural information of ghrelin precursor is of importance in understanding it function. From our results, we found that the solution structure of ghrelin is mostly random coil conformation at neutral pH value and the structural population changes with pH environments. Data from circular dichroism in different TFE concentrations revealed that the secondary structure changes from random coil to α-helix and the isodichroic point is observed at 202nm, implying that two equilibrium states exist between random coil and helical structure.
Keywords : Growth factor hormone, Pro-ghrelin, NMR, Circular dichroism

INTRODUCTION

Through the past research, it was well known that the ghrelin functions as agonists of orphan GHSR.[1] One of the research groups found that the ghrelin not only stimulates growth hormone but also increases appetite.[2] Therefore, it was considered as a target molecule for obese treatment.[3] Additionally, it was demonstrated that the ghrelin regulates insulin signaling and immune system based on previous reports.[4-6] Recently, it has been shown that the ghrelin has effects on hormone balance and directly involved in homeostasis adjustment of immune-systems and food up-take level.[7-10]

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The ghrelin precursor is secreted in the inner wall of stomach and is modified from the precursor to mature form by post transcriptional modification. In the past research, it had not been surely reported which enzyme acts on this modification process specifically.[11-13] But later, academic research group noticed that pro-hormone convertase 1/3 co-localizes with the ghrelin in stomach and have an important role in specific cleavage between Arginine-28 and Alanine-29 of the pro-ghrelin.[4] In addition, the GOAT (Ghelin O-Acyltransferase) was considered as an important enzyme to modify the ghrelin Serine-3 by O-acylation (Fig. 1).[14]

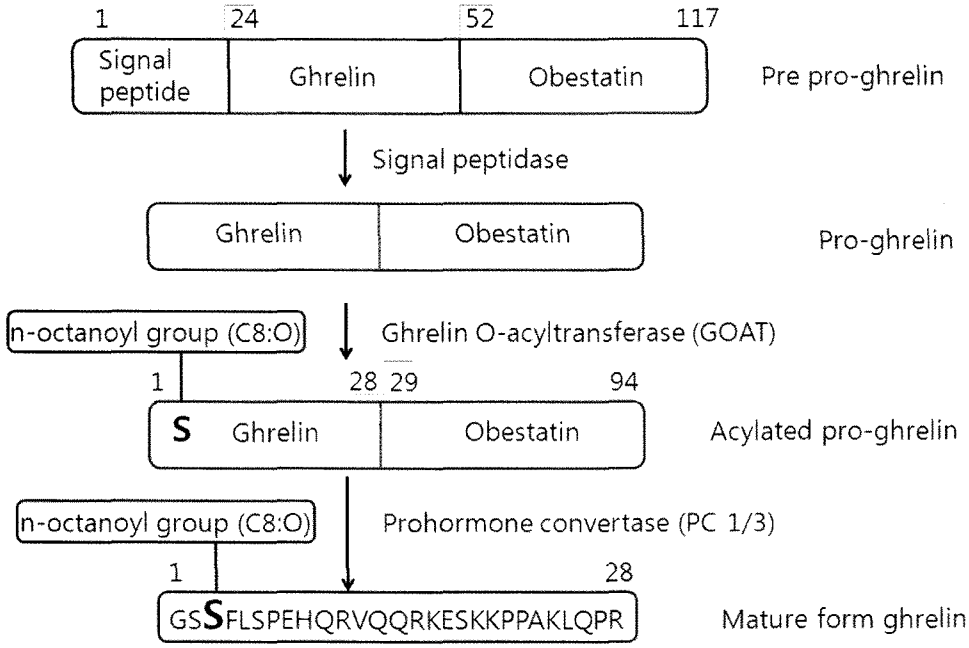


Fig. 1. The post-transcriptional modification processes from the pre pro-ghrelin to mature ghrelin. Mature ghrelin have 28 amino acid residues and serine-3 is a conserved acyl-modification site. Acyl-transmodification step by GOAT comes before the cleavage step.by PC1/3.

Although the ghrelin is a very important and unique peptide hormone in many metabolic processes, the structural information is still unknown. To understanding its biological role in the structural level, the structure study of pro-ghrelin is essential. However, in many

cases, the precursor of hormone peptides does not have precise folded structure until to be mature form. Pro-ghrelin is a 94-residues protein with a molecular weight of 10.6 kDa, and the pI is 5.68. It was cloned and expressed through several biochemical methods and purified by using nickel affinity column and gel filtration. We performed NMR experiments and circular dichroism to characterize its structure in various buffer conditions.

EXPERIMENTAL

Pro-ghrelin Construct Cloning and Purification

Using human cDNA as a template, Pro-ghrelin gene was obtained by PCR and inserted into the pET-15b. This vector contained His_6-tag and Thrombin cleavage site. In cloning process, TEV recognition site was added with pro-ghrelin gene. All sub-cloning work was performed in *E.coli* DH5α strain. Pro-ghrelin was over-expressed in *E.coli* BL21 (DE3) strain (Invitrogen Inc.). Especially, the unique 7-amino acid residues (GRIFLQD) were involved in N-terminal region for the purpose of increasing protein solubility. However, solubility of pro-ghrelin was not so high. Hence finding out optimal cell culture conditions was required.

For the over-expression of pro-ghrelin in *E.coli*, 0.4 mM isopropyl β-D-thiogalactoside (IPTG) was added to LB and M9 medium when the optical density of cell at 600 nm reached 0.45~0.5 and it transferred from 37℃ to 20℃ incubator. For soluble expression, low temperature and low shaking rpm were required.

Cells were harvested after 20hr overnight culture by centrifugation at 6000 x *g* for 30 minutes. Cell pellet was melted and lysed by the sonicator in the binding buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, in pH 8.0). Soluble supernatant was separated from the precipitate using ultra centrifuge (14000 x *g* for 25 min) and it was loading in Ni^{2+} affinity chromatography (Amersham Pharmacia).

The column were washed twice (10x column bed volume) with 25 mM imidazole washing buffer and eluted with 350 mM imidazole elution buffer. His_6-tag in N-terminal region was removed by TEV cleavage site and sample buffer was exchanged by gel filtration chromatography (Superdex 75).

Sample Preparations for NMR Spectroscopy

Sample for NMR experiments were 0.5 mM concentration in 100 mM sodium chloride, 0.01% NaN_3, 20 mM HEPES buffer pH values of 7.0 in 90% H_2O/10% ^2H_2O solution. NMR sample in 70% H_2O/30% 2, 2, 2-trifluoro-(d3)-ethanol (TFE) mixture (v/v) at pH 7.0 was prepared after lyophilization of a sample in the aqueous solution.

Sample Preparation for Circular Dichroism

For the pH titration assay, All pro-ghrelin samples were prepared in various buffer by buffer exchange method using PD-10 desalting column; in 70 mM glycine buffer with pH range of 2.5 – 3.5, 70 mM sodium acetate with pH range of 4.0 – 5.0, 35 mM sodium citrate with pH range of 5.5 – 6.5, and 35 mM Tris buffer with pH range of 7.0 – 9.0. The concentration of all samples was 50 μM in this time. For the sample preparation in different concentration of TFE, protein samples were diluted to 50 μM concentrations and TFE were mixed in protein samples as 10-50% in 20 mM HEPES buffer at pH 7.0.

NMR Experiments and Data Processing

NMR spectra were recorded at 298 K on Bruker DRX-500 MHz spectrometer equipped with a triple-resonance probe with x,y,z-gradients. 1D-^1H and 2D-[^1H-^{15}N] HSQC NMR experiments were performed with the different buffer conditions (in 0%, 30% TFE) and pulsed field gradient techniques with a WATERGATE pulse sequence were used for the good suppression of the solvent signal.[15,16] Spectra were processed with the NMRPipe/nmrDraw software package and analyzed using the Sparky program.[17,18]

Circular Dichroism

CD spectra of Pro-ghrelin were measured at 25℃ on a Jasco 810 spectropolarimeter. Far-UV CD spectra were monitored from 190-250 nm using the protein concentration of 50 μM with a path length of 0.1 cm, a response time of 1 s, and a scan speed of 50 nm/min. Spectra were recorded as an average of five scans.

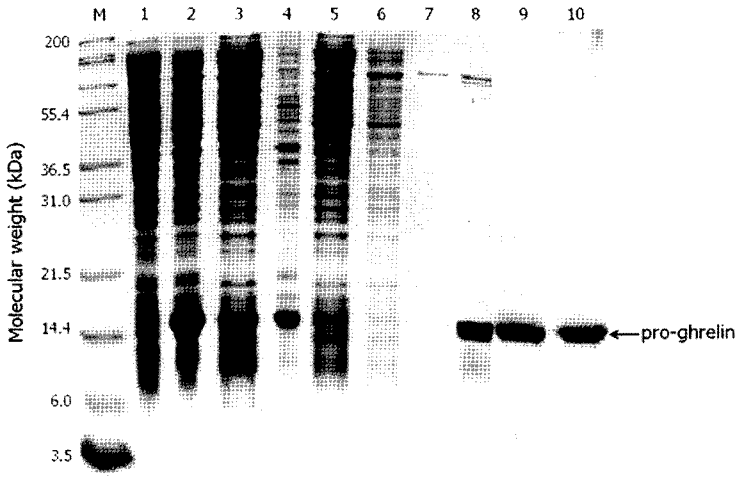
RESULTS AND DISCUSSION

Pro-ghrelin was cloned with TEV cleavage site and chaperon-like functional peptide sequence in pET15b vector encoding His_6-tag and thrombin site. So expressed protein size was higher than real size. In SDS PAGE, purified pro-ghrelin band was presented near the 14.4 kDa of the marker, and the purity was over than 90 percent (Fig. 2A). After cleavage reaction, N-terminal region including His_6-tag, thrombin and TEV cleavage site, and chaperon-like peptide was removed. The molecular weight of the final protein is 10.6 kDa. In the gel filtration profile shows the native protein as a dimer. X is a running volume reflecting from highest peak and Y is a protein size and it can be obtained by substitution X value in this formula ($\log^{y}$= -0.0249x + 3.1429). It is suggested that pro-ghrelin exists as a dimer form in our buffer condition (Fig. 2B). From the 1D-^{1}H NMR spectrum of pro-ghrelin at 298 K, we confirmed that protein is correctly folded by chemical shift dispersion, showing that two tryptophan residues were observed near 10ppm (Fig. 3A).

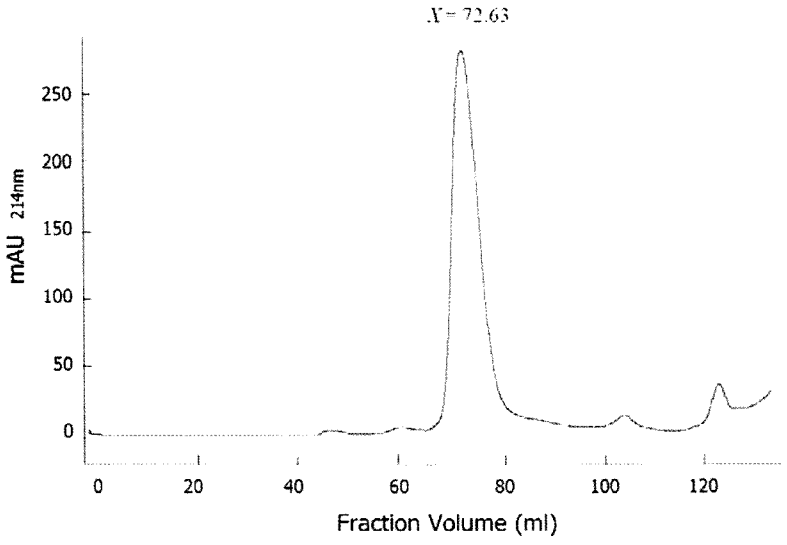
2D-[^{1}H-^{15}N] HSQC data of pro-ghrelin indicated that pro-ghrelin have a mostly random coiled form based on its chemical shift dispersion (Fig. 3B). Secondary structure was also confirmed and analyzed by CD experiment, suggesting that pro-ghrelin possess a high percentage of random coil structure in solution state, however the pH value does not effect on conformational change in secondary structure (Fig. 5A).

Since TFE is an inducer that transforms the random coil to α-helix in partially folded protein, it is useful for characterization in partially folded structure. Therefore, we applied TFE to observe conformational change of pro-ghrelin.[19,20] As the TFE concentration increases, typical α-helix pattern were built up, which means that TFE leads pro-ghrelin structure to the high α-helix content. There are no significant change between 30 and 50% TFE in CD spectra, implying that overall conformation does not change significantly beyond 30% TFE. We also found the isodichroic point at 202nm which means only two states, α-helical and random coiled conformations, were existed at each TFE concentration (Fig. 5B).

We also confirmed the conformational change in the presence of TFE by NMR spectroscopy. There were significant changes in 2D-[^{1}H-^{15}N] HSQC spectrum. A number of



(A)



(B)

Fig. 2. SDS-PAGE analysis for purification step. (A) Purification of His_6-tags pro ghrelin. Lane (1), Before induction; (2), after induction; Lane (3), supernatant after cell lysis; Lane (4), pellet after cell lysis; Lane (5), flow through-out of Ni-NTA column; Lane (6), first column washing; Lane (7), second column washing; Lane (8 – 10), elution. (B) Gel filtration profile on superdex 75 and SDS-PAGE result of each fraction. According to this formula $\log^{y}$= -0.0249x +3.1429 (x=column running volume (ml)), pro-ghrelin formed soluble dimer.

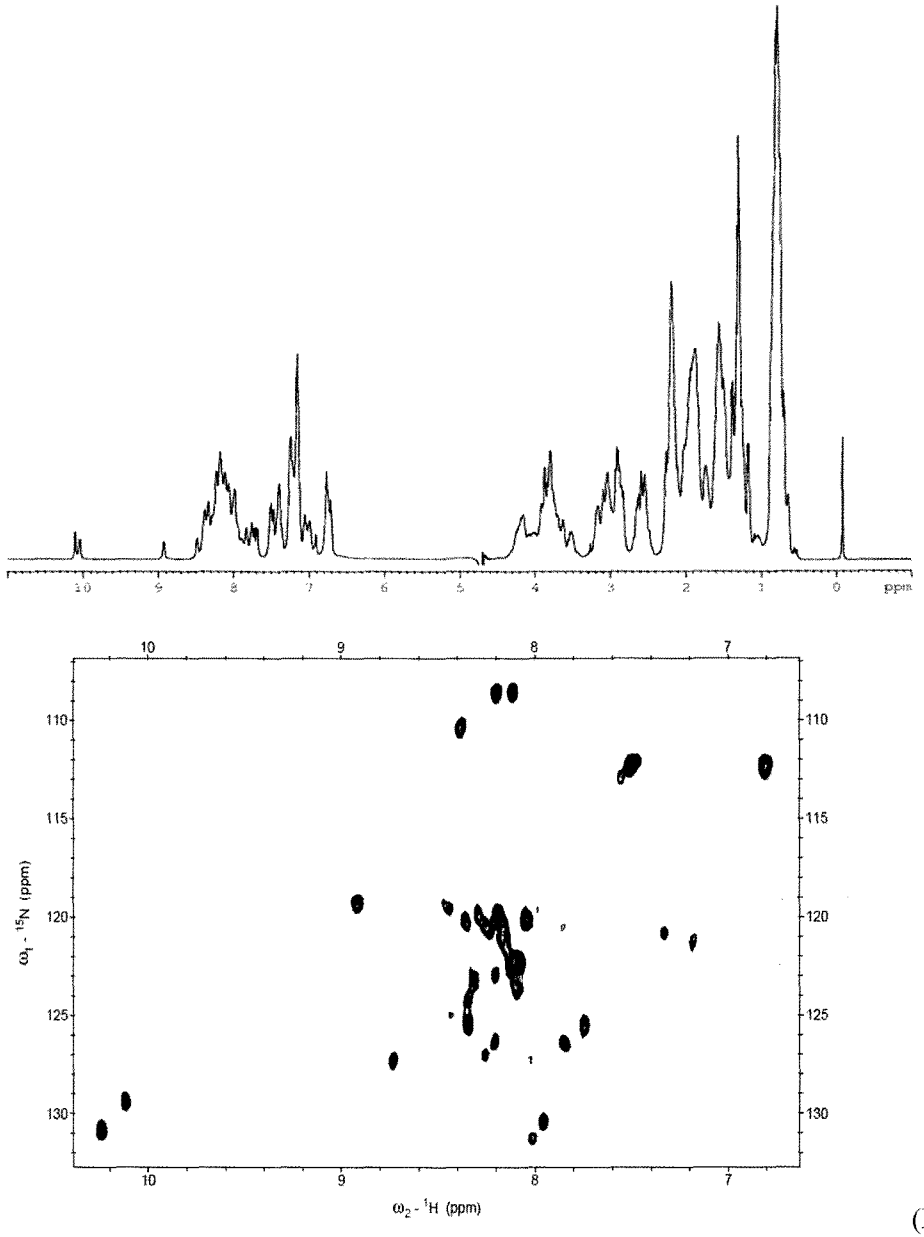


Fig. 3. (A) One-dimensional ^{1}H-NMR spectrum, and (B) 2D-[^{1}H-^{15}N] HSQC spectrum of pro-ghrelin in 20 mM HEPES condition at pH 7.0. The Spectrum was acquired at pH 7.0 and 298 K using Bruker DRX-500.

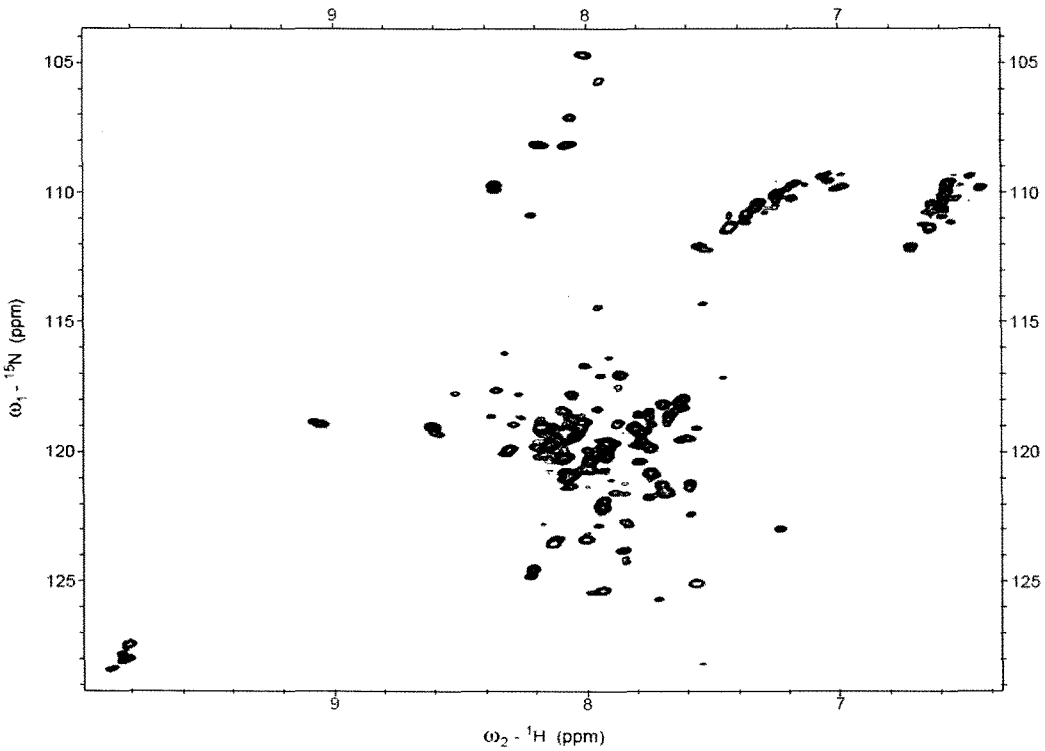
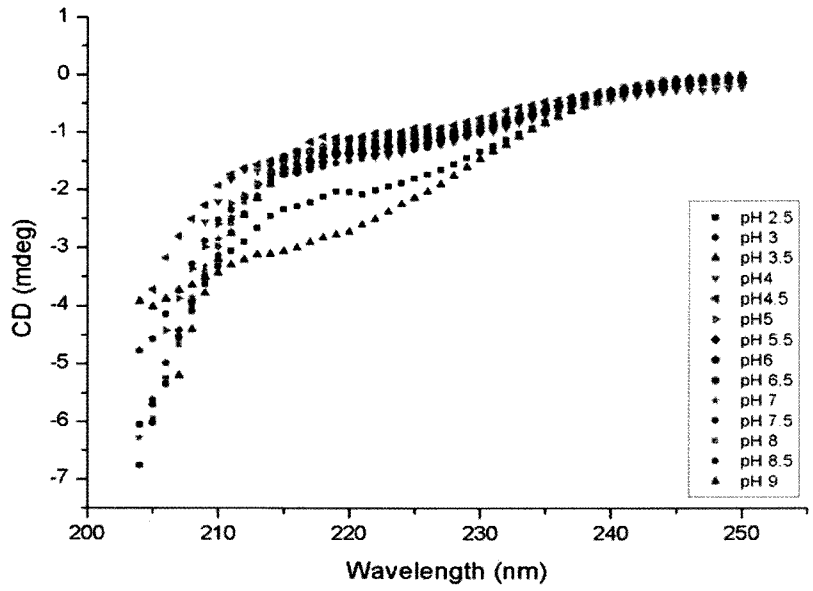


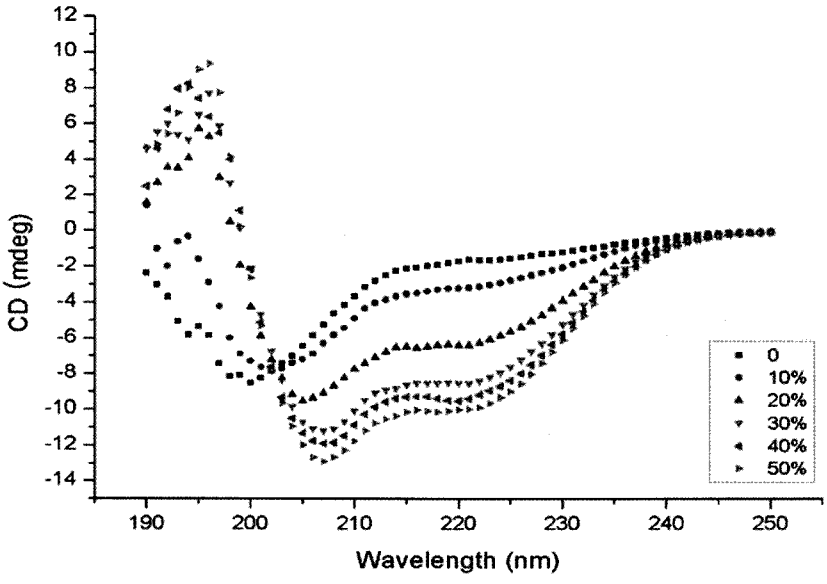
Fig. 4. 2D-[^{1}H-^{15}N] HSQC spectra of pro-ghrelin in 20 mM HEPES, 30% TFE at pH 7.0. It was recorded on Bruker DRX-500 MHz spectrometer at 25℃.

residues were shifted in the 2D-[^{1}H-^{15}N] HSQC spectrum. However, the spectra pattern shows that the protein possesses both random coil and helical structure. Therefore, it is considered that α-helical secondary structure is formed by TFE, however, the tertiary structure was not formed well (Fig. 4).

In this study, the conformational change of pro- ghrelin was examined using NMR and CD. Even if, we could not obtain the detailed structural information, our data will provide the useful information for the study of tertiary structural transition via molecular interaction such as protein- ligand and protein-protein within a cell.



(A)



(B)

Fig. 5. Far-UV CD spectra of pro-ghrelin in various conditions. CD spectra (A) in different pH value, (B) in different concentrations of trifluoroethanol (TFE) at 25℃. The pH factor didn't effect on protein structural changes. Otherwise, the change of TFE concentration (TFE>30%) is effective.

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