^{15}N labeling method of peptides using a thioredoxin gene fusion expression system: an application to ACTH-(1–24)

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Abstract For structure analysis of peptides by multinuclear NMR, stable isotope-labeled samples are required. A direct over-expression system by *E. coli* cells does not work for that purpose because of rapid degradation of the peptides and/or the mRNA in host cells. We here developed an over-expression system by means of thioredoxin gene fusion system. The fused protein composed of thioredoxin and the objective peptide was expressed in *E. coli* and then the peptide part was released by enterokinase. This system was successfully applied for the production of ^{15}N-labeled human adrenocorticotropic hormone fragment (ACTH-(1–24)) as needed for multinuclear NMR analysis.

Key words: Human adrenocorticotropic hormone; Thioredoxin; Enterokinase; Nuclear magnetic resonance

1. Introduction

In recent years, the experimental techniques of the multinuclear and multidimensional NMR have drastically progressed. The most important role of such experiments is to reduce signal degeneracy in higher molecular weight proteins. Although there are several advantages (such as structure analysis of peptides complexed with their receptors or receptor fragments, heteronuclear chemical shift analysis, or quantitative ^3J coupling constant measurement, etc.) in those experiments besides from better signal separation, there are few cases applied to peptides so far. This may be the result of the difficulty of stable isotope labeling of peptides with ^{15}N and/or ^{13}C. Because the chemical synthesis of labeled peptides is prohibitively expensive, an over-expression system, in other words, a biosynthesis system is required for this purpose. However, an over-expression system designed to express the objective peptide directly would suffer from the very rapid degradation of the peptides and/or the mRNA in host cells such as *E. coli*.

We have been interested in the relationship of structure and function of human adrenocorticotropic hormone fragment 1–24 (ACTH-(1–24)) that is a bioactive fragment of the originally 39 membered peptide and an anterior pituitary origin [1]. Although we analyzed its solution structure using chemically synthesized and non-labeled sample, its C-teminal region did not converge into a single conformation [2]. In order to have much more detailed structure information, we needed stable isotope labeled ACTH-(1–24), in other words, a realistic over-expression system of ACTH-(1–24) because of reasons described above. Then, we constructed an over-expression system to biosynthesize relatively short peptides aimed for heteronuclear NMR analysis. In this system, the objective peptide is expressed as a fused protein with thioredoxin and released with a sequence specific protease. We here report an application to uniform ^{15}N-labeling of ACTH-(1–24) as well as its over-expression system.

2. Materials and methods

2.1. Construction of ACTH-(1–24) expression vector

To construct an expression system, two expression vectors, pMMPB2 [3] and pTRX-FUS [4] were joined (Fig. 1). The thioredoxin gene containing a multicloning site at the 3′ end was amplified by PCR with the plasmid pTRX-FUS as template with the following two oligonucleotides as primers containing *Eag*I, *Nde*I and *Hin*dIII restriction enzyme sites, respectively (these sites are underlined in the primer sequence):

(1) GCCCCCGGCCG CATATGAGCGATAAAATTATTCACC

(2) GCCCCGAAGCTTCACCCTGTACGATTACTGCAGGTCG

The PCR product was digested with *Eag*I and *Hin*dIII and ligated into the *Eag*I and *Hin*dIII backbone fragment of pMMPB2. To create an expression vector pTRF plasmid, the purified plasmid containing such fragments was digested with *Nde*I restriction enzyme and then self-ligated. Plasmid pTRX-ACTH was generated by ligating a synthetic DNA fragment encoding the ACTH-(1–24) amino acid sequence (dAGCTACAGTATGGAGCACTTCCGCTGGGGCAAGCCCGT-AGGCAAGAAGCGACGACCCGTAAAGGTATACCCCTAGCT-GCA/dGCTAGGGGTATACCTTTACGGGTCGTCGCTTCTTGC-CTACGGGCTTGCCCCAGCGGAAGTGCTCCATACTGTAGCT) into the *Kpn*I(blunted)–*Hin*dIII backbone fragment of pTRX plasmid.

2.2. Expression and purification of ACTH-(1–24)

The plasmid pTRX-ACTH was transformed into the host strain BL21(DE3) cell [5]. The transformed cells were grown in 2 l YT medium at 37°C and then expression was initiated by IPTG (final concentration 1 mM) when cells had grown to 0.6 OD (620 nm). After 3 h cells were harvested and frozen below −20°C. The frozen cell paste was suspended in 5 ml lysis buffer (20 mM Tris-HCl, pH 8.0, 2.5 mM EDTA)/1 g wet cells followed by sonication for 20 min on ice. Insoluble material was removed by centrifugation at 20,000 × *g* for 30 min at 4°C. The supernatant was then loaded onto a HiTrap SP column (Pharmacia Biotech) pre-equilibrated with buffer A (20 mM Tris-HCl, pH 8.0, 1 mM EDTA). The protein was eluted with a linear gradient from 50 mM to

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Abbreviations: NMR, nuclear magnetic resonance; *E. coli*, *Escherichia coli*; HSQC, heteronuclear single quantum coherence spectroscopy; SDS-PAGE, sodium dodecyl sulfate polyacryl amide gel electrophoresis; RP-HPLC, reverse phase high pressure liquid chromatography; TFA, trifluoroacetic acid; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TPPI, time proportional phase increment; IPTG, isopropyl-β-D-(−)-thiogalactopyranoside.

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1 M KCl in buffer A. The fused protein was located in the 0.1–0.2 M KCl fraction and dialyzed against distilled water. The purity of the isolated protein was analyzed by 20% SDS-PAGE.

The purified thioredoxin/ACTH-(1–24) fused protein was digested overnight with the sequence specific protease, enterokinase (Funakoshi) in the digestion buffer (70 mM succinate pH 5.6, 0.5 mM $CaCl_2$) at 25°C. After digestion, RP-HPLC was used to isolate ACTH-(1–24). For this purpose TFA was added to the digested sample (final concentration of TFA was 0.1%). The solution was loaded onto a C18 column (Nakarai) pre-equilibrated with buffer A (90% water, 10% acetonitrile, 0.1% TFA), and elution was carried out with a linear gradient of buffer B (90% acetonitrile, 10% isopropylalchol, 0.1% TFA).

2.3. *N-Terminal analysis and amino acid analysis of the purified ACTH-(1–24)*

The N-terminal sequence of the ACTH-(1–24) was determined by the Edman degradation method. Amino acid analysis was performed with a Hitachi 835-S amino acid analyzer on the acid hydrolysates (6 N HCl containing 0.2% phenol, 110°C, 24 h).

2.4. *Expression and purification of uniformly ^{15}N-labeled ACTH-(1–24)*

^{15}N uniformly labeled ACTH-(1–24) was expressed and purified following the protocols described above with slight modifications: M9 minimum medium was used instead of YT medium, 2 g of $^{15}NH_4Cl$ was added to 2.5 l medium as ^{15}N source before the cell growth was initiated. Upon induction with IPTG cell growth proceeded overnight. After RP-HPLC fractions containing ^{15}N-labeled ACTH-(1–24) were lyophilized, then dissolved in distilled water, adjusted to pH 6 and lyophilized again.

2.5. *NMR experiments*

The ^{15}N-labeled ACTH-(1–24) was dissolved in aqueous 95% 2,2,2-trifluoroethanol-d_2 (Isotech's product). 2D-HSQC [6,7,8] experiments were carried out on a Bruker's ARX500 spectrometer at 500 MHz for proton frequency in the temperature range from 279 K to 288 K. Spectral width for the ^1H axis was set at 6250 Hz and that of ^{15}N at 330 Hz, respectively. A GARP pulse sequence was used for ^{15}N decoupling during acquisition [9]. 500 μs of spin locking purge pulse was applied just before the second ^1H 90 degree pulse in the HSQC sequence in order to suppress the hydroxyl proton signal from trifluoroethanol [10]. Data for HSQC spectrum with 32 (^{15}N) × 512 (^1H) in the complex-sized time domain were acquired with TPPI-States method [11] for the indirect detection of ^{15}N in order to achieve phase sensitive mode spectra.

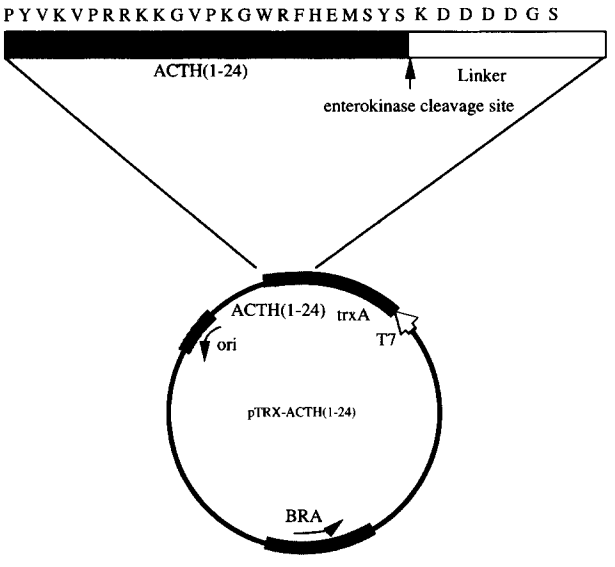

Fig. 1. Schematic illustration of the plasmid pTRX-ACTH-(1–24). The amino acid sequence of ACTH-(1–24) and the linker sequence which is recognized by enterokinase are shown in one letter code. The enterokinase recognition sequence is indicated in bold and the cleavage site with an arrow.

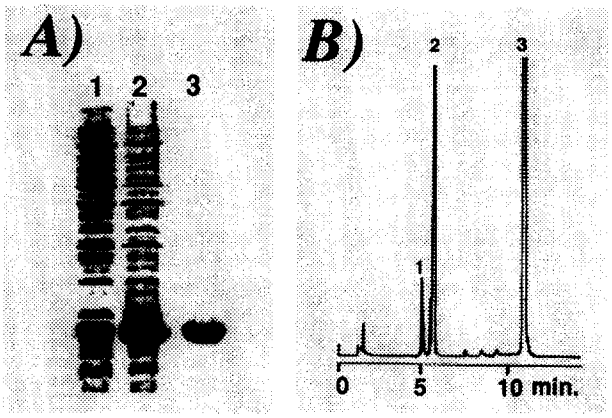

Fig. 2. (A) Purity of the thioredoxin fused ACTH-(1–24) peptide as analyzed by 20% SDS-PAGE; lane 1: lysate of uninduced BL21(DE3) containing pTRX-ACTH; lane 2: lysate of induced BL21(DE3) containing pTRX-ACTH; lane 3: purified thioredoxin fused ACTH-(1–24). (B) The results of reverse-phase HPLC as described in section 2. ACTH-(1–24) was found in peak 2.

3. Results

The expression vector pTRF for the N-terminal fragment 1–24 of ACTH (Fig. 1) was constructed for an over-expression vector by joining two expression vectors, i.e. pMMPB2 which contains T7 promoter and pTRX-FUS which contains the thioredoxin fused gene so that the thioredoxin gene is controlled by the T7 polymerase expression system. Over-production of the thioredoxin/ACTH-(1–24) fused protein was obtained by adding IPTG (1 mM) to BL21(DE3) cells with the plasmid pTRF-ACTH-(1–24) at the mid-logarithmic phase. 3 h after the induction, the content of the fused protein was estimated to be 40% of the total cell protein by SDS-polyacrylamide gel electrophoresis (Fig. 2A). The harvested cells were lysed by sonication and the fused protein was detected in the soluble fraction. After ion exchange chromatography high purity (95%) the thioredoxin/ACTH-(1–24) fused protein was isolated in good yield (ca. 10 mg from 1 l of culture) (Fig. 2A). The fused protein was digested with enterokinase, a protease which recognizes the amino acid sequence Asp-Asp-Asp-Asp-Lys. Then, ACTH-(1–24) was isolated by RP-HPLC (Fig. 2B). Peak 1 and peak 2 (Fig. 2B) obtained from this chromatography were analyzed for their N-terminal sequence and amino acid composition. The N-terminal sequence analysis indicated peak 1 as a mixture of two peptides mixture; one with Trp-Gly-Lys-Pro-Val and the other with Ser-Tyr-Ser-Met-Glu at the N-termial. On the other hand, the N-terminal sequence of peak 2 was Ser-Tyr-Ser-Met-Glu. The latter two peptide sequences corresponded to the N-terminus of ACTH-(1–24), whereas the Trp-Gly-Lys-Pro-Val sequence corresponds to the amino acid sequence 9–13 of ACTH-(1–24). To confirm that the peak 2 peptide is ACTH-(1–24) an amino acid analysis was performed (Table 1). In addition, the chemically synthesized ACTH-(1–24) (gift of J.W. van Nispen) showed the same retention time as peak 2 (data not shown).

Uniformly ^{15}N-labeled ACTH-(1–24) was obtained along with almost the same protocol as decribed above. We obtained ca. 6 mg of ^{15}N-labeled ACTH-(1–24) per 1 l M9 culture medium. Fig. 3 shows the region of the cross section in amide

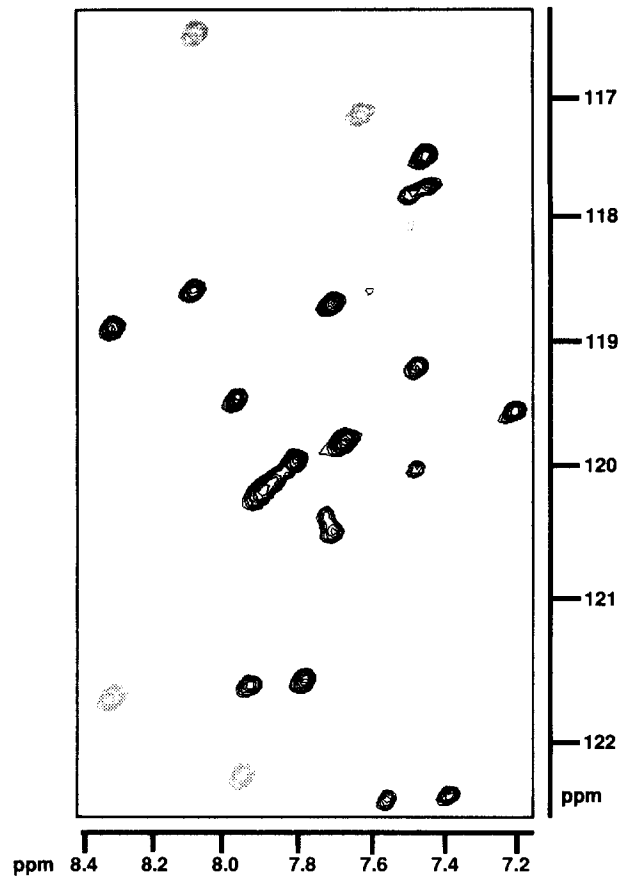

Fig. 3. ^{15}N–^{1}H HSQC (heteronuclear single quantum coherence spectroscopy) spectrum of ^{15}N-labeled ACTH-(1–24) in 95% aqueous TFE-d_2.

proton and amide nitrogen of the ^{15}N–^{1}H HSQC spectrum of ACTH-(1–24) in 95% aqueous 2,2,2-trifluoroethanol-d_2 at 15°C.

4. Discussion

The thioredoxin gene-fused expression system, originally constructed by LaVallie [4], bears the main advantage that thioredoxin as a periplasm protein, when expressed in *E. coli.*, is translocated to the periplasm and accumulated at high concentration. This feature is advantageous for expression of peptides in *E. coli* since the thioredoxin-fused system serves to prevent digestion by endogenous proteases. In the original expression system, the expression of the target protein is controlled by *trp* promoter, therefore, tryptophan is needed to induce the target protein. However, when a labeled protein is needed, the *trp* promoter is not good choice because the tryptophan has to be added to the medium for the induction. This added tryptophan is metabolized by *E. coli* and taken up into the target protein. Therefore as a result, the target protein cannot be labeled uniformly by the stable isotopes, ^{15}N or ^{13}C. To overcome this problem, we switched the promoter sequence from the *trp* promoter to the T7 promoter. The T7 promoter has the advantage that IPTG is used instead of tryptophan to induce the expression of recombinant proteins.

It is said that the thioredoxin-fused system has the advantage of facile purification because thioredoxin is stable to 80°C and allows for heating the cell lysate at 80°C for a few minutes. Although this heating procedure could be applied in our case, it caused almost half the thioredoxin-fused ACTH-(1–24) to precipitate into the debris fraction. Therefore, we used conventional ion chromatography for separation and purification. As shown in Fig. 2A, the purity of the thioredoxin-fused ACTH-(1–24) was sufficiently high for isolation and the recovery rate was higher than in the heat treatment procedure (data not shown).

Protease (enterokinase) digestion was used to release ACTH-(1–24) from the fused protein. For this purpose two different buffer conditions were used, i.e. Tris-HCl buffer at pH 8.0 and succinate buffer at pH 5.6. Fig. 2B shows the results of the digestion in the succinate buffer at pH 5.6 as analyzed by HPLC. Two main peaks and one minor peak were observed. Even though a highly purified enterokinase was used for this experiment, it contained trypsin contaminant. Consequently, peak 1 resulted from trypsin digestion. This was confirmed with Tris-HCl buffer at pH 8.0 where peak 1 increased because trypsin activity is higher at pH 8.0 (data not shown). This biosynthetic material behaved identically to chemically synthesized material in amino acid analysis and chromatography.

Finally, ^{15}N–^{1}H HSQC spectra (Fig. 3) were measured and compared with the amide-alpha proton region of 2D-NOESY spectra. Because the peptide contains 3 Pro residues, 21 ^{15}N–^{1}H cross-peaks were expected, but significantly more peaks were observed. This may be attributed to conformational equilibria involving *cis–trans* isomerization of prolyl residues. Since the cross-peaks in the 2D-NOESY spectra of unlabeled ACTH-(1–24) were largely overlapping and thesensitivity of the NOESY spectra was significantly lower than that of the HSQC spectra as expected for peptides of this size, in fact, these additional cross-peaks could not be found in the homonuclear experiments. This shows the benefits of such an uniform labeling procedure in small peptides. Generally the intensities of NOE cross peaks are small for peptides with molecular weight in the range of 1000–5000. Because the sequece specific resonance assignment procedure of non-labeled peptides or proteins depends on NOESY spectra, it has been sometimes difficult to assign ^{1}H resonances as well as to elucidate the structure of such peptides using interatomic distances derived from NOE intensities. Once isotope labeling has been done, triple resonance experiments can be easily employed for making sequential

Table 1
The result of amino acid analysis[a]

Amino acid	Amount (nmol)	Normalized composition[b]	Predictided composition from DNA
Lys	8.839	3.80	4
His	2.357	1.01	1
Arg	7.055	3.04	3
Ser	4.822	2.08	2
Glu	2.425	1.04	1
Pro	5.937	2.56	3
Gly	5.158	2.22	2
Val	6.691	2.88	3
Met	2.269	0.98	1
Tyr	4.643	2.00	2
Phe	2.323	1.00	1

[a]The amount of Trp was not determined.
[b]The calculated composition was normalized to Phe as one.

assignment of backbone atoms by means of well resolved heteronuclear one-bond and two-bond couplings. Furthermore, secondary structure preferences can be derived directly from J-coupling constants and from chemical shift differences from the standard values without using NOE's (manuscript in preparation). Thus efficient over-expression systems as that described in this study may prove to be useful for obtaining more precise conformational informations on bioactive peptides in a variety of environments including those complexed with their receptors.

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