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Application of Temperature Gradient Gel Electrophoresis To cAMP Receptor Protein

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Cyclic AMP receptor protein (CRP) is involved in the transcriptional regulation of more than 100 genes in *E. coli*. CRP dimer is converted into active form via the sequential conformation change of cAMP binding pocket, hinge region and HTH DNA binding motif by binding of cAMP. The temperature gradient gel electrophoresis (TGGE) was applied to CRP protein to know whether it was an efficient technique to study the conformational transitions and the thermal stability. TGGE showed the unfolding process of wild-type and S83G CRP proteins with the temperature gradient set from 29 to 71℃ on nondenaturing polyacrylamide gel. Melting temperature (Tm) was 57±1 and 55±1℃ for wild-type and S83G CRP, respectively in acidic buffer[89.8 mM Glycine and 24 mM Boric acid (pH 5.8)].

Key words – Cyclic AMP receptor protein; Protease digestion; Tm (melting temperature); Protein denaturation

Every protein plays a specific role with a unique conformation. Despite a number of papers dealing with protein structures, the correlation between protein structure and function is not yet sufficiently understood. The folding/unfolding process of any protein is of great importance for the understanding of structure-function relationship. The conformational transition can be induced by changing temperature, adding denaturants, varying pH, applying high pressure, or adding ligands[5]. The studies on the conformational transitions of proteins help to broaden understanding of the folding process in cell after translation, the mechanism of regulatory conformational changes, and the formation of active conformation for the biologically engineered protein[13].

Many publications[2,4,10,13,14,16] reported results about conformational transitions, thermal stability, or melting temperature (Tm) of proteins using temperature gradient gel electrophoresis (TGGE). This technique has an advantage in revealing all fractions of protein sample within the temperature range during the same time. And changes in protein properties, such as charge and conformational transition, are translated into a change of mobility[16].

Cyclic AMP receptor protein (CRP), which acts as homodimer, is well known protein which regulates transcription of many genes in *E. coli*[1,7]. Each subunit of CRP is made up of amino-terminal domain, which contains cAMP binding pocket surrounded by a β-roll structure and a very long α-helix, and carboxy-terminal domain, which provides helix-turn-helix motif to bind DNA [8]. CRP is converted into active conformation by binding of cAMP to its binding pocket and its complex activates the transcription expression by binding to its target site with not fully understood mechanism.

In this study, TGGE was applied to CRP to know whether it is an efficient technique to study the conformational transitions and the thermal stability for the dimeric protein. And also it was used for S83G CRP, which substituted glycine at 83 for serine[11]. In addition, CRP conformation was studied at the various temperatures by the proteolytic digestion.

Materials and Methods

Materials

CRP was purified from the strain CA8445/pRK248 (cI^{ts}) harboring recombinant pRE2*crp* plasmid according to the methods by Harman *et al.*[8] except the following procedure. The Affi-gel Blue gel chromatography (Bio-Rad) was used further to eliminate the high molecular weight contaminants. The proteins were higher than 95% purity by 12.5% SDS-PAGE. Chymotrypsin, Glycine, Boric acid, CM Sephadex, and Coomassie brilliant R were purchased from Sigma. GB buffer used in this study contains 89.8 mM Glycine and 24 mM Boric acid (pH 5.8 at 25℃). CRP

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concentrations were determined using the extinction coefficient 3.5×10^4 $M^{-1}cm^{-1}$ at A_{280} nM[11].

Temperature gradient gel electrophoresis

TGGE unit consists of two electrode tanks and aluminum heating blocks with two water baths connected to provide a temperature gradient perpendicular to the electrophoretic migration[4]. A 6% polyacrylamide gel (37.5 : 1, acrylamide:bisacrylamide) was used. The temperatures of two water baths were set at 20 and 90℃ but the actual gel temperatures were actually measured by DP 465 thermometer with a needle-like thermocouple probe (TQ, Omega Inc). The temperature gradient on the gel was also linear and uniform within the region covered by the aluminum heating block (data not shown).

The protein migration was directed from top (+) to bottom (-) due to the positive net charge of CRP (pI, 9.16) in GB buffer (pH 5.8). Each gel was run at 200 volts for 2.5hrs and stained with Coomassie brilliant blue. The gel was dried with Hoefer Gel Dryer and then scanned with microdensitometer to measure band intensity. The measured data at each point were used to plot the fraction of denatured protein against temperature to determine the melting temperature (Tm).

Chymotrypsin digestion

Protease digestion reactions were carried out as described by Harman *et al.*[8] at a volume of 30 μl at 37℃ in GB buffer (pH 5.8). The ratio of CRP to chymotrypsin was 200 : 1 by weight. Reactions were terminated by addition of PMSF to final concentration of 5 mM. Peptides were resolved by electrophoresis on 20% polyacrylamide-SDS gels and visualized by staining with Coomassie brilliant blue.

CD spectra measurement

CD data were obtained at range of wavelength from 190 nM to 320 nM by Jasco 710 spectrapolarimeter (Basic Science Institute) with path length of 0.1 mM. Measurements were conducted at 0.3 mg/ml of each CRP at either 25 or 55°C in GB buffer (pH 5.8).

Results and Discussion

In TGGE, if the temperature gradient is established in the gel perpendicular to the direction of electrophoresis, the protein molecules will adopt a temperature-dependent conformation according to their positions in the temperature gradient. Protein molecules with different conformations can be separated if native or denatured conformations have an altered hydrodynamic volume[16]. There are several requirements that TGGE can be applied to proteins[4]. First, pH of electrophoresis buffer should be lower or higher than isoelectric point (pI) of the protein to migrate through the native gel. Second, pH of the buffer should be independent of the gel temperature to avoid pH change by gel temperature during the electrophoresis.

The protein migration is directed from top (+) to bottom (-) due to the positive net charge of CRP (pI, 9.16) in GB buffer (pH 5.8). Fig. 1 shows the results of TGGE for wild-type and S83G CRP proteins. Each figure shows two bands; the lower band for the native CRP protein and the upper band at high temperature for the denatured CRP protein with the retarded migration. Several publications[2,14,16] showed that the denatured proteins migrated slowly and accumulated to precipitates easily. Malecki *et al.*[12] proposed that CRP is denatured from dimer through native monomer to denatured monomer by the unfolding and refolding kinetics of CRP. It could not be verified that whether the band between dimer and denatured monomer stands for native monomer or any intermediate conformation during the unfolding process.

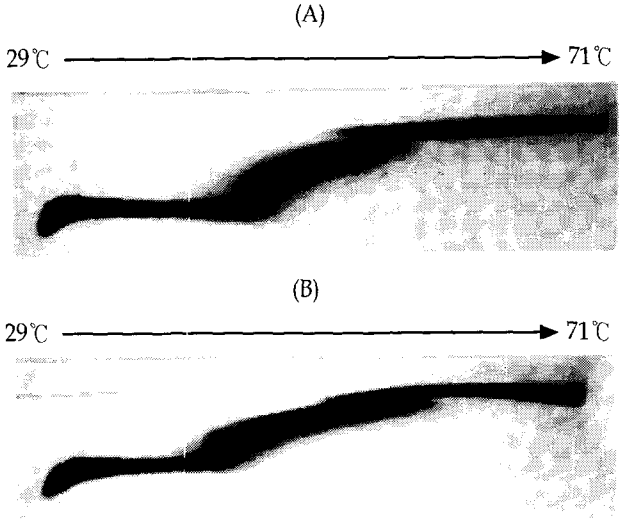

Fig. 1. TGGE profiles of wild-type and S83G CRP at the final concentration of 50 μM in GB buffer. CRP migrated from top (+) to bottom (-). Temperature gradient was set from 29℃ to 71℃. It was perpendicular to the direction of protein migration. Reaction condition was mentioned in Methods and Materials.

To estimate the thermal stability or Tm of CRP, the scanning microdensitometer was used to measure the band intensity of Fig. 1 at each temperature. Fig. 2 showed the graphs corresponding to the denatured fraction against temperature. Each denatured fraction is the ratio of band intensity for denatured monomer at each position to that for the maximal monomer around 71℃. Band intensity was determined by the integration of same size of area at each position. Fig. 2 shows the good linear relationship between the denatured fractions and gel temperatures. Tm values are 57±1 and 55±1℃ for wild-type and S83G in GB buffer at pH 5.8, respectively. Shi *et al.*[15] measured Tm's of 64.9±0.1℃ at pH 6.0 and 57.7±0.1℃ at pH 5.2 for wild-type CRP by Differential Scanning Calorimeter in high salt buffer. pH of GB buffer used in this study was -0.59 at 50℃ relative to that at 20℃[16]. It means that the actual pH of solution was ~5.2 for the protein migrating at 50℃. In addition, UV spectrophotometric measurement showed that Tm of CRP was 57.9℃ in the same buffer[9]. It suggested that Tm values measured by TGGE were very consistent with those determined by other biochemical methods[9,15].

To figure out the protein conformation around the melting temperature estimated by TGGE, proteolytic digestion and CD measurement were conducted at several temperatures. In Fig. 3, wild-type and S83G CRP proteins were digested by chymotrypsin either in the presence or absence of cAMP at 37, 45, and 55℃. Chymotrypsin produced mainly α-CRP in all CRP with cAMP but in S83G CRP low molecular peptide fragments, which were degraded randomly and converted into the smaller fragments compared to α-CRP(shown at high temperature). Baker *et al.*[3] showed that the position where protease cleaves CRP was dependent upon the stability of the CRP dimer. If CRP was stable, then the cleavage occured in the hinge region (around position 135). But if it was unstable, the cut occured in the c-helix around position 116. In

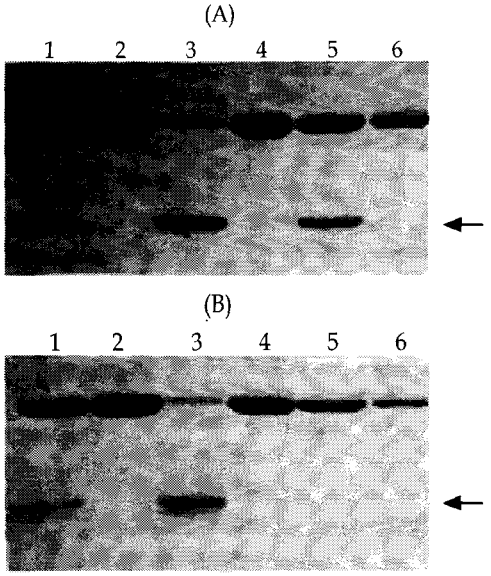

Fig. 3. Proteolytic digestion of wild-type (A) and S83G CRP (B) proteins by chymotrypsin. Each lane contained CRP either in the presence (2, 4, and 6 lanes) or absence of cAMP (1, 3, and 5 lanes). 50 μM of cAMP concentration was used in this study. Reactions were conducted in GB buffer at 37℃ (1 and 2 lanes), 45℃ (3 and 4 lanes), and 55℃ (5 and 6 lanes). The digested α-CRP is indicated with arrow.

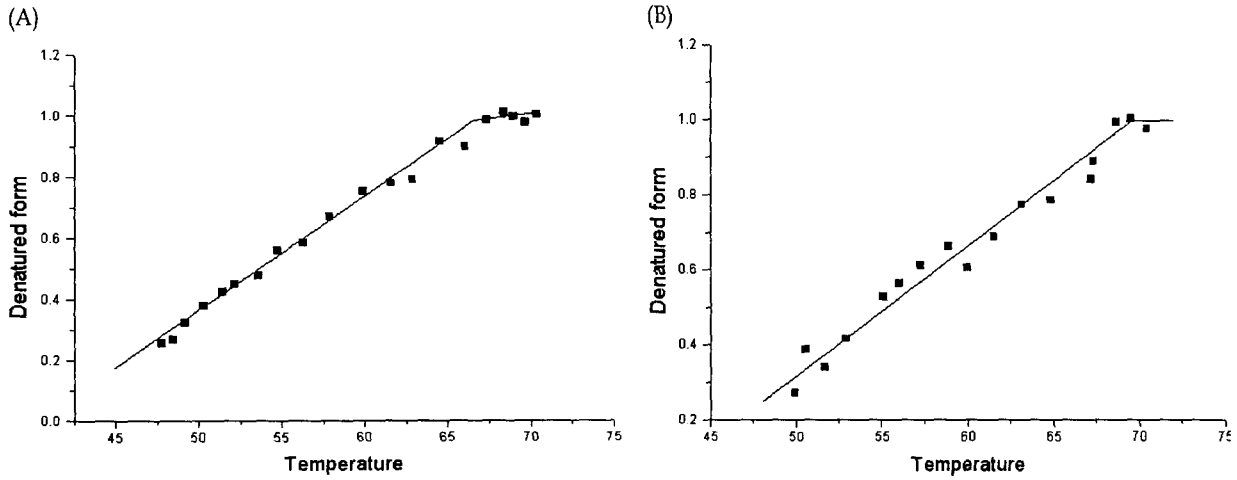

Fig. 2. Plots of the relative fraction of the denatured form of wild-type (A) and S83G (B) CRP proteins against temperature. The relative fraction was obtained from Fig. 1 by measuring band intensity with microdensitometer. Tm was determined at the denatured fraction of 0.5.

addition, the α-CRP of S83G CRP was shown little in the presence of cAMP at 55℃(shown in Fig. 3-(B)). It suggested that cAMP molecules were dissociated from α-CRP at this temperature. Lee *et al*[11] reported that S83G CRP had somewhat lower affinity for cAMP than did wild-type CRP and cells that contained the S83G CRP: cAMP complex supported only half the β-galactosidase activity of cells that contained the wild-type CRP:cAMP complex. The hydroxyl group of serine was supposed to be involved in a weak hydrogen bonding to the oxygen moiety of cAMP. When cAMP was released, α-CRP was completely degraded into small pieces by protease[6].

CD data were obtained at wavelength from 190 to 320 nM by Jasco 710 spectrapolarimeter with path length of 0.1 mM. Typical CD spectra of wild-type and S83G CRP at 25℃ and 55℃ above 250 nM are shown, respectively, in Fig. 4 and 5. Below 250 nM at 25℃ and 55℃, the CD spectra are little different, which indicates no large change in α-helical structure of both proteins at 55℃. The CD spectra of S83G CRP above 250 nM exhibit changes compared to those of wild-type CRP at 25℃ and 55℃, resulting from the different sensitivity of the tertiary structure to temperature. Especially, the absorption peaks at 295 nM are different for both protein at 25℃ and 55℃, which can be interpreted that the tertiary structure around the two tryptophan residues in the amino-terminal domain is changed as the temperature is increased from 25 ℃ to 55℃. And S83G CRP shows high structure sensitivity to temperature with the comparison to that of wild-type CRP in Fig 6.

This result suggests that TGGE can be applied to study the denaturation processes and the thermal stability of any protein. And it can be also applied to the diagnosis of diseases which is related to the protein mutation

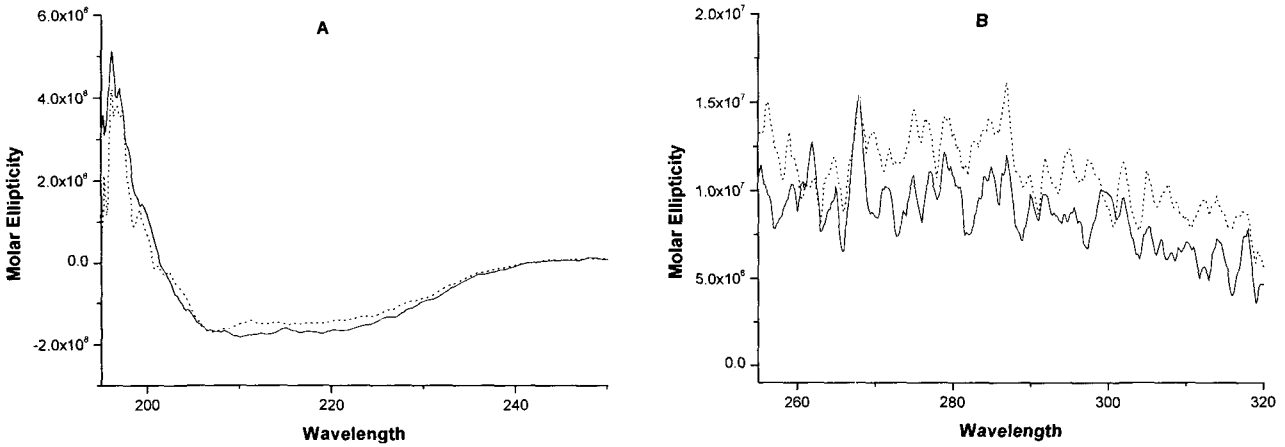

Fig. 4. Far-UV CD spectra (top) of wild-type CRP at 25℃ (—) and 55℃(---). Near-UV CD spectra(bottom) of wild-type CRP at 25℃ (—) and 55℃ (---). CRP was used at 0.3 mg/ml.

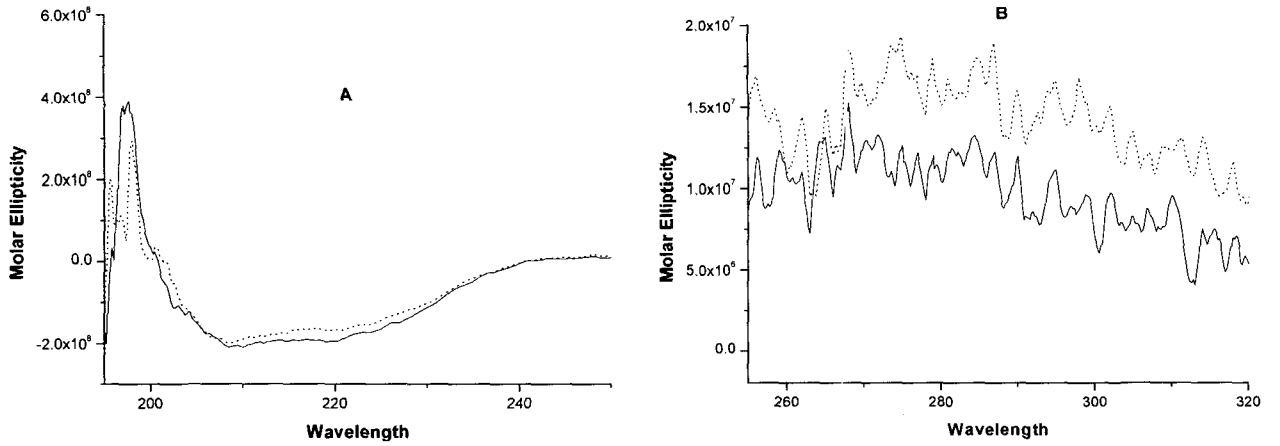

Fig. 5. Far-UV CD spectra (top) of S83G CRP at 25℃ (—) and 55℃ (---). Near-UV CD spectra (bottom) of S83G CRP at 25℃ (—) and 55℃ (---). CRP was used at 0.3 mg/ml.

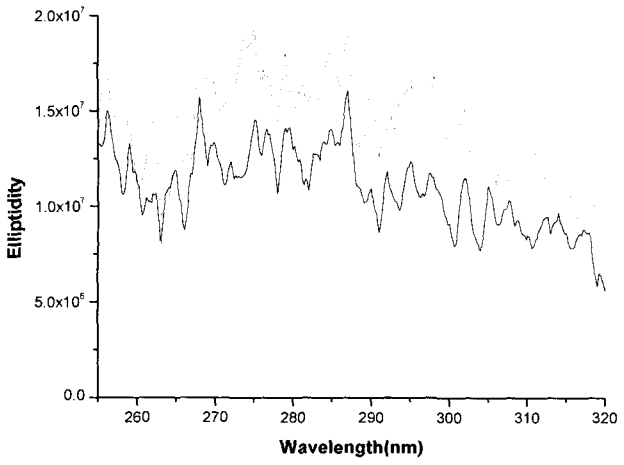

Fig. 6. Near-UV CD spectra of wild-type (—) and S83G (---) CRPs at 55℃. Graph was plotted from data of Fig. 4 and 5 to show the temperature sensitivity of CRP structure.

resulted in the different conformation.

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References

1. Aiba, H., S. Fujimoto and N. Ozaki. 1982. Molecular cloning and nucleotide sequencing of the gene for *E. coli* cAMP receptor protein. *Nucleic Acid Res.* **10**, 1345-1350.
2. Arakawa, T., L. Hung, V. Pan, T. P. Horan, C. G. Kolvenbach and L. O. Narhi. 1993. Analysis of the heat-induced denaturation of proteins using temperature gradient gel electrophoresis. *Anal. Biochem.* **208**, 255-259.
3. Baker, C. H., S. R. Tomlinson, A. E. Garcia and J. G. Harman. 2001. Amino acid substitution at position 99 affects the rate of CRP subunit exchange. *Biochemistry* **40**, 12329-12338.
4. Birmes, A., A. Sattles, K.-H. Maurer and D. Riesner. 1990. Analysis of the conformational transitions of proteins by temperature-gradient gel electrophoresis. *Electrophoresis* **11**, 795-801 .
5. Blundell, T. L., B. L. Sibanda, M. J. Sternberg and J. M. Thornton. 1987. Knowledge-based prediction of protein structures and the design of novel molecules. *Nature* **326**, 347-352.
6. Botsford, J. L. and J. G. Harman. 1992. Cyclic AMP in prokaryotes. *Microbiological Reviews* **56**, 100-122.
7. Cossart, P. and B. Gicquel-Sanzey. 1982. Cloning and sequence of the crp gene of *Escherichia coli* K 12. *Nucleic Acids Res.* **10**, 1363-1378.
8. Harman, J. G.., K. McKenney and A. Peterkofsky. 1986. Structure-function analysis of three cAMP-independent forms of the cAMP receptor protein. *J. Biol. Chem.* **261**, 16332-16339.
9. Joo, J., M.-J. Goo and J. Gang. 2000. Study on the structure of cAMP receptor protein (CRP) by temperature change. *Korean Journal of Life Science* **10**, 279-285.
10. Koch, M., R. Wahl and F. J. Seif. 1995. Analysis of thyroid stimulating hormone-receptor mutations by temperature-gradient gel electrophoresis. *Electrophoresis* **16**, 742-743.
11. Lee, E. J., J. Glasgow, S.-F. Leu, A. O. Belduz and J. G. Harman. 1994. Mutagenesis of the cyclic AMP receptor protein of *Escherichia coli*: targeting positions 83, 127 and 128 of the cyclic nucleotide binding pocket. *Nucleic Acids Res.* **22**, 2894-2901.
12. Malecki, J. and Z. Wasylewski. 1997. Stability and kinetics of unfolding and refolding of cAMP receptor protein from *Escherichia coli*. *Eur. J. Biochem.* **243**, 660-669.
13. Rosenbaum, V. and D. Riesner. 1987. Temperature-gradient gel electrophoresis. Thermodynamic analysis of nucleic acids and proteins in purified form and in cellular extracts. *Biophys. Chem.* **26**, 235-246.
14. Sattler, A. and D. Riesner. 1993. Temperature-gradient gel electrophoresis for analysis and screening of thermostable proteases. *Electrophoresis* **14**, 782-788.
15. Shi, Y., S. Wang and F. P. Schwarz. 2000. Intersubunit association induces unique allosteric dependence of the T127L CRP mutant on pH. *Biochemistry* **39**, 7300-7308.
16. Viglasky, V., M. Antalik, J. Bagel'ova, Z. Tomori and D. Podhrasky. 2000. Heat-induced conformational transition of cytochrome c observed by temperature gradient gel electrophoresis at acidic pH. *Electrophoresis* **21**, 850-858.

초록 : 온도 기울기 전기영동장치의 CAMP 수용성 단백질에 응용

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cAMP 수용성 단백질(CRP)은 *E. coli*의 100가지 이상의 유전자 전자조절에 관계된다. CRP는 dimer로 존재하며 cAMP의 결합으로 활성인 형태로 전환된다. 이중체인 CRP 단백질의 열 안정성과 구조 전이의 연구에 효과적인 온도 기울기 전기영동장치를 이용하여 확인하였다. 본 연구에서 야생형과 S83G CRP 단백질의 melting temperature (Tm)는 산성인 완충용액[89.8 mM Glycine, 24mM Boric acid (pH 5.8)]에서 57±1(야생형 CRP)과 55±1℃(S83G CRP)였다. 그리고 온도에 따른 CRP 단백질의 구조변화도 protease digestion과 CD spectropolarimeter을 이용하여 확인하였다.