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# Determination of protein phosphorylation by extracellular signal-regulated kinase using capillary electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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**Abstract**

Extracellular signal-regulated kinase (ERK) is a key regulatory enzyme mediating cell responses to mitogenic stimulation and is one of the key components in linking growth factor receptor activation to serine/threonine protein phosphorylation processes. Phosphorylation reaction by ERK plays an important role in many signal transduction pathways. ERK phosphorylates numerous substrates such as MBP, microtubule-associated protein 2 (MAP2) and nuclear protein. In particular, MBP is a substrate commonly employed for the detection of ERK activity and contains the consensus primary sequence $PRT_{97}P$. In this paper, we compared the degree of the phosphorylation reaction of MBP substrate peptides by ERK with the three different MBP substrate peptides, MBP1(KNIVTPRTPPPSQGK), MBP2(VPRTPGGRR) and MBP3(APRTPGGRR) in order to select an efficient substrate peptide for phosphorylation reaction by ERK. The results showed that the MBP3 peptide is the most efficient substrate for phosphorylation reaction by ERK. Using MBP3 peptide, the phosphorylation reaction of MBP by ERK was monitored with both matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and capillary electrophoresis (CE). Our results demonstrate the feasibility of the CE method, the method being a simple and reliable technique in determining and characterizing various kinds of enzyme reaction especially including kinase enzymes.


*Keywords:* Enzymes; Proteins; Extracellular signal-regulated kinase

## 1. Introduction

Protein phosphorylation, which is an important type of protein modification, is a key mechanism for regulating intracellular proteins including such crucial activities such as cell differentiation and proliferation, gene expression, and a number of metabolic processes [1]. Many cellular processes are regulated by phosphorylation reaction induced conformational changes and activation of proteins [2]. The enzymes involved in phosphorylation reaction can be classified into two broad classes, tyrosine kinases and serine/threonine kinases. They phosphorylate the respective amino acid residue in protein employing phosphate donors such as adenosine triphosphate (ATP). The detection of phosphorylation reaction is an important step in the investigation of signal transduction pathways [3].

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Mitogen-activated protein kinase (MAPK), which is a serine/threonine kinase, is a key regulatory enzyme mediating cell responses to a variety of extracellular stimuli, such as hormones, interleukines, mitogenic factors, etc. [4]. Extracellular signal-regulated kinase (ERK), which is one of the MAPK families, is a key regulatory enzyme mediating cell responses to mitogenic stimulation and is activated in response to peptide growth factors and phorbol esters. Activated ERK plays an important role in many signal transduction pathways by phosphorylating numerous substrates including MBP, microtubule-associated protein 2 (MAP2) and nuclear protein [5]. Especially, MBP has been shown to be an effective substrate for ERK. The specific sequence in bovine MBP phosphorylated by ERK was identified as Thr97 in the target substrate, $PRT_{97}P$ [6].

In the past, studies of ERK have been extremely difficult because of its low absorbance and because it requires the use of [$^{32}$P]phosphate labeling of phosphoproteins to be visualized on 2-D polyacrylamide gels. This method has disadvantages such as being time consuming, expensive and requiring difficult handling [7,8].

Recently, CE has become a powerful analytical system for the detection of a wide variety of molecules from small organic molecules to macro molecules such as DNA and protein [9]. CE has also been used as a method for enzyme assays [10–12] including protein kinase assay since a CE-based protein kinase assay was established by Dawson et al. [13].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF-MS) is a versatile and sensitive technique in the analysis of a variety of large biomolecules including mixtures of high molecular mass proteins, enzymatic protein digests, oligosaccharides, nucleic acids, and some biomolecules in biological fluids [14]. In addition to CE, MALDI–TOF-MS has often been used to identify phosphopeptides [15] and to monitor protein kinase assays [16,17]. MALDI–TOF-MS also offers high sensitivity and the ability to process multiple samples quickly [18].

In this paper, we investigated the degree of the phosphorylation reaction by ERK according to the sequence of the MBP substrate peptides. The used MBP substrate peptides were MBP1-(KNIVTPRTPPPSQGK), MBP2(VPRTPGGRR) and MBP3(APRTPGGRR). For this experiment, we first studied analysis conditions such as the pH of electrolyte and concentration of acetonitrile in order to detect simultaneously the substrate and product peptides. Using the obtained conditions, we compared phosphorylation reaction by characterization of the three MBP substrate peptides and selected the most efficient substrate peptide for phosphorylation reaction by ERK. Using the most effective substrate for phosphorylation reaction by ERK, we observed the phosphorylation reaction in various amounts of enzyme units using CE. Also, we confirmed the phosphorylation reaction of MBP substrate peptide by ERK using MALDI–TOF-MS.

## 2. Experimental

### 2.1. Chemicals and reagents

ERK was obtained form New England Biolabs (Beverly, MA, USA). The three MBP substrate peptides were from Anygen (Gwangju, South Korea). Trizma hydrochloride, β-glycerol phosphate, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid (EGTA), sodium orthovanadate, dithiothreitol (DTT), magnesium chloride, adenosine triphosphate (ATP), acetonitrile (ACN), methanol, α-cyano-4-hydroxycinnamic acid (α-CHCA) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma (St. Louis, MA, USA). The water used for sample preparations was obtained from a Milli-Q plus water purification system from Millipore (Molsheim, France). All the samples were passed through a 0.45-μm membrane filter unit and carefully degassed before use.

### 2.2. Capillary electrophoresis and sample preparation

The CE instrument employed was a Waters Quanta 4000 CE system from Waters (Milford, MA, USA) equipped with a 185-nm wavelength mercury lamp UV detector from Waters. CE analysis was performed using 150 m*M* Tris–phosphate buffer containing 5% acetonitrile, pH 2.8, in untreated fused-

silica capillary columns of effective length 27 cm× 75 μm I.D. from Polymicro Technologies (Phoenix, AZ, USA). The samples were injected hydrodynamically for 30 s. The applied voltage was 8 kV and the current was 80 μA. The electropherograms were recorded on a Waters 746 data module. The shaking water bath was from Vision (Seoul, South Korea).

### *2.3. MALDI–TOF-MS and sample preparation*

MALDI–TOF-MS analysis was performed on an HP G2025A instrument from Hewlett-Packard (Palo Alto, CA, USA) equipped with a 1-m linear type time-of-flight mass spectrometer and a pulsed nitrogen laser (337-nm radiation). This instrument was operated in the positive-ion detection mode. Typically, the spectra from 20 shots were summed to obtain the final spectrum.

α-CHCA and DHB were used for MALDI–TOF-MS matrix. α-CHCA matrix solution was prepared by mixing 33 m*M* α-CHCA with acetonitrile–methanol–water (5:3:2, v/v). DHB matrix solution was prepared by mixing 100 m*M* DHB with acetonitrile–water (1:1, v/v). A 0.5-μl aliquot of the sample mixture was applied on the target followed by a 0.5-μl aliquot of matrix solution. The solution was then left to dry at room temperature, inserted into the mass spectrometer and analyzed.

### *2.4. Incubation conditions for the detection of the phosphorylation reaction by ERK*

The phosphorylation reaction mixture was prepared by mixing 5-μl of MBP peptides (0.1 mg/ml), 5 μl of ATP–$MgCl_2$ cocktail (500 μ*M* ATP and 75 m*M* $MgCl_2$ in kinase buffer) and 5-μl of kinase buffer (20 m*M* Tris–HCl buffer, pH 7.2, containing 25 m*M* β-glycerol phosphate, 5 m*M* EGTA, 1 m*M* DTT, 1 m*M* sodium orthovanadate). The reaction was initiated by adding ERK. Assays were performed at 30 °C for 30 min.

## 3. Results and discussion

Phosphorylation of protein is an important step in many cellular regulatory processes such as in the signal transduction pathways. One aspect of understanding signal transduction and its modulation by phosphorylation is the degree to which these enzymes recognize their substrates and the degree to which they act on those substrates. There was increasing need for substrates to be studied in relation to the sequence of certain signaling proteins. In our study, we investigated the phosphorylation reaction by ERK with different three MBP substrate peptides in order to obtain the most capable substrate for phosphorylation reaction. The sequence and characterization of the three used MBP substrate peptides are shown in Table 1.

First, we investigated the optimal conditions for MBP substrate peptides using CE in order to study the phosphorylation reaction by ERK. We used the acidic electrolyte buffer to decrease adsorption, substrate peptides's basic nature, between the capillary inner wall and the sample. A 150 m*M* Tris–phosphate buffer with a pH range from pH 2.0 to 3.0 was used to find the best conditions for the analysis of the MBP substrate peptides. In this result, we observed that the sensitivity and migration time of each peptide was increased according to the increase in the buffer pH. Thus, at pH 2.8, fairly good sensitivity and migration time were obtained. Although the use of a pH 3.0 could improve the sensitivity, the lengthened analysis time is inadequate because enzyme assay using CE should generally be carried out in a short time [19]. Theoretically, one would expect that when the pH of the running buffer is raised, the electroosmotic flow increases and migration time decreases [20]. However, in our experiment, instead of a decrease in the migration time of the tested substrate peptides triggered by an increase in the pH of the running buffer from 2.0 to 3.0, a reverse trend was observed. This observation could be explained as follows: at low pH in this range, the ionization of surface silanol groups in the capillary column is suppressed and the electroosmot-

Table 1
Characterization of MBP substrate peptides

| | Structure of peptide | $M_r$ | Isoelectric point |
|---|---|---|---|
| MBP1 | KNIVTPRTPPPSQGK | 1620.01 | 11.17 |
| MBP2 | VPRTGGRR | 995.30 | 12.30 |
| MBP3 | APRTGGRR | 967.20 | 12.30 |

ic flow approaches zero, therefore the analytes migrate mainly by electrophoretic flow instead of electroosmotic flow. Therefore, we selected 150 m*M* Tris–phosphate buffer, pH 2.8, as the electrolyte buffer.

Also, we tested the effect of the addition of acetonitrile in the running buffer for the detection of the three MBP substrate peptides. Although acetonitrile is not required for MBP substrate peptides analysis, we used acetonitrile due to its effects on increasing the sensitivity and decreasing the current when a running buffer of high concentration, such as 150 m*M*, is used. Fig. 1 shows the CE electropherogram of the MBP substrate peptides according to different concentrations of acetonitrile. In this experiment, we found that an increase in acetonitrile concentration induced an increase in sensitivity of the three MBP substrate peptides, while the resolution of substrate peptide and other material peaks decreased. Especially, when the MBP2 peptide was detected in the electrolyte buffer containing 10% acetonitrile, the MBP2 peptide peaks appeared close to other material peaks (Fig. 1B). Therefore, the Tris–phosphate buffer containing 5% acetonitrile was determined to be the optimal condition. In conclusion, the analysis optimal condition for detection of the three MBP peptides is 150 m*M* Tris–phosphate buffer containing 5% acetonitrile at pH 2.8, and 185-nm UV detection.

Using the obtained conditions obtained from the above investigation, we compared phosphorylation reaction by characterization of the three MBP substrate peptides to decide the best substrate for the phosphorylation by ERK using CE. Fig. 2 shows the electropherogram of the ERK reaction mixtures without and with 10 U/ml ERK, respectively. Each phosphorylation reaction mixture was prepared by mixing 0.1 mg/ml MBP substrate peptides, ATP–$MgCl_2$ cocktail and kinase buffer together. The reactions were started by adding ERK at 30 °C for 30 min. The degree of the phosphorylation reaction by ERK was identified from the decrease in the substrate peaks. In this result, compared with other peptides, the decrease in MBP3 peptide peaks was

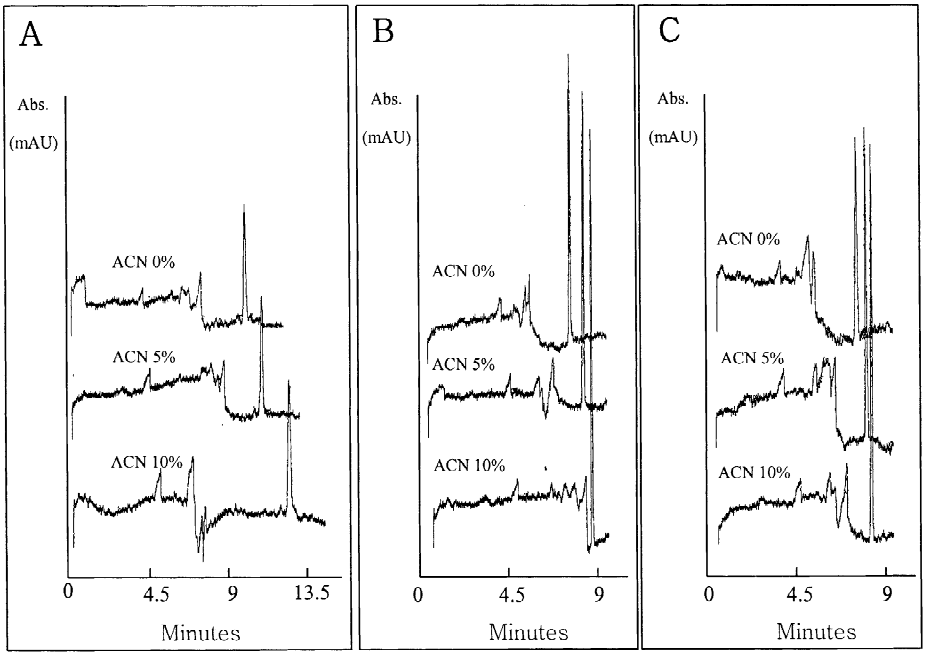


Fig. 1. CE electropherogram of the MBP substrate peptides by concentration of acetonitrile. (A) MBP1 peptide, (B) MBP2 peptide, (C) MBP3 peptide. Each substrate was diluted in kinase buffer (20 m*M* Tris–HCl buffer, pH 7.2, containing 25 m*M* β-glycerol phosphate, 5 m*M* EGTA, 1 m*M* DTT, 1 m*M* sodium orthovanadate). CE conditions: 75 μm×35 cm untreated fused capillary column, 150 m*M* Tris–phosphate buffer containing various acetonitrile volume (pH 2.8), 8 kV, 185-nm UV detector, injection time 20 s.

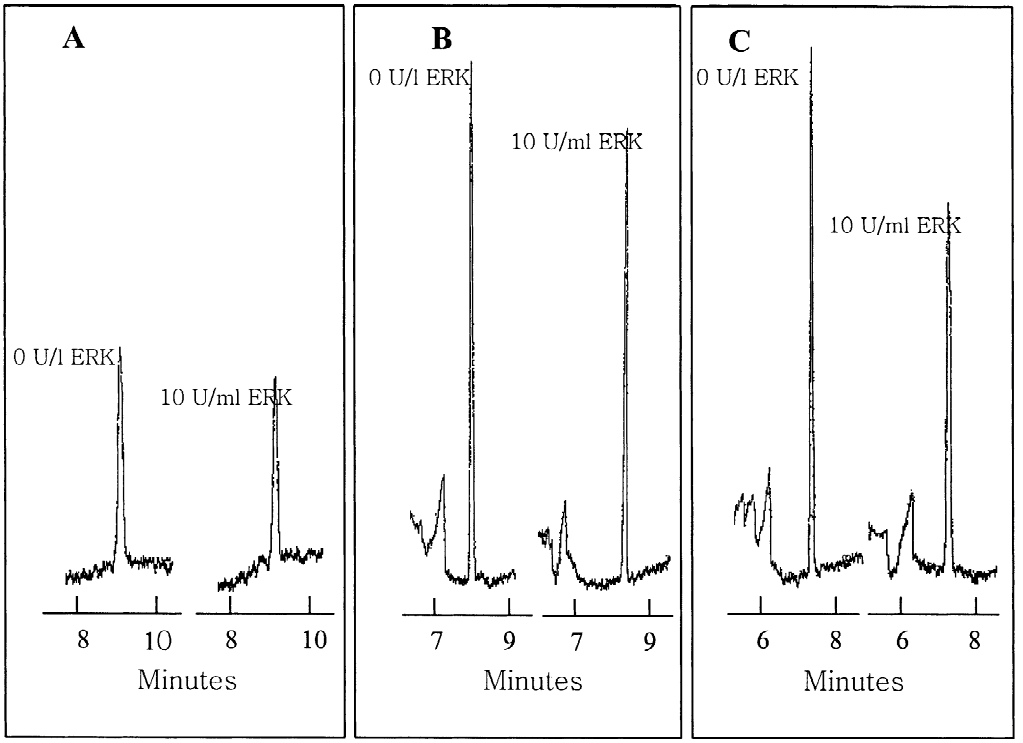


Fig. 2. CE electropherogram of the ERK reaction mixture without or with 10 U/ml enzyme. (A) MBP1 peptide, (B) MBP2 peptide, (C) MBP3 peptide. The phosphorylation reaction was carried out in a mixture of 0.1 mg/ml MBP substrate peptides, ATP–$MgCl_2$ cocktail and kinase buffer by adding ERK at 30 °C for 30 min. Other CE conditions are the same as in Fig. 1 except the 150 m*M* Tris–phosphate buffer containing 5% acetinitrile, pH 2.8.

observed to be the largest (Fig. 2C). Therefore, we selected the MBP3 peptide as the effective substrate for the phosphorylation reaction by ERK.

Using MBP3 peptide, the effect of the amount of ERK enzyme on the phosphorylation of MBP was investigated. The reactions were carried out within the range 0–40 U/ml of ERK with limited MBP3 peptides. The dependence of the phosphorylation reaction on the amount of enzyme can clearly be seen in Fig. 3. As the amount of ERK increased, the MBP3 peptide peak decreased and converted to phosphorylated MBP3 peptide peak. However, the increase in amount of phosphorylated MBP3 peptide was not distinctly visible at amounts of enzyme greater than 30 U/ml. This is probably because the enzyme does not act as a catalyst in the reaction since it is saturated with limited substrate. Fig. 4 shows the CE electropherograms of the phosphorylation reaction of MBP3 peptide with 0, 20 and 40

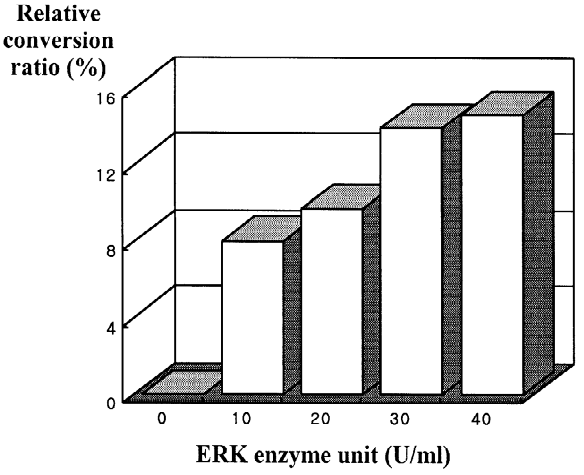


Fig. 3. Influence of amount of enzyme on phosphorylation reaction by ERK. The phosphorylation reaction conditions and CE conditions are the same as in Fig. 2.

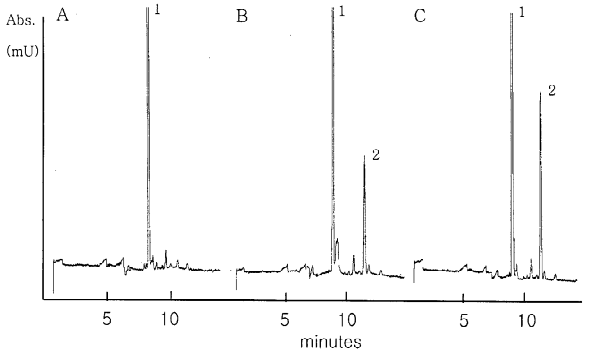


Fig. 4. CE electropherograms of the phosphorylation reaction of MBP3 peptide with ERK. (A) 0 U/ml ERK, (B) 20 U/ml ERK, (C) 40 U/ml ERK. The phosphorylation reaction conditions and CE conditions are the same as in Fig. 2.

U/ml ERK. The phosphorylated MBP3 peptide peak appeared with the addition of ERK at 10.4 min. According to the increase in the ERK amount, we observed that the MBP3 peptide peak was decreased and the phosphorylated MBP3 peptide peak was increased (Fig. 4B and 4C). In this result, the addition of 20 and 40 U/ml of ERK in the reaction mixture resulted in about 9.8 and 14.7%, respectively, conversion of the MBP3 peptide to phosphorylated MBP3 peptide.

In order to confirm the phosphorylation reaction obtained from the above investigation, we also carried out mass analysis of the reaction mixture after phosphorylation reaction by ERK using MALDI–TOF-MS. We used α-CHCA and DHB as the MALDI matrix for the phosphorylation reaction mixture. When we used α-CHCA as the matrix for MBP3 substrate peptide, we observed signals stronger than that of DHB. Therefore, we decided to use α-CHCA as the matrix for this study. Fig. 5 shows the MALDI–TOF-MS mass spectra of the phosphorylation reaction mixture without and with 20 U/ml ERK. The molecular ion peak in the MALDI–TOF-MS spectrum consists of the $[M+H]^+$ ion. As shown in Fig. 5A, when the phosphorylation reaction was carried out without ERK, the only MBP3 peptide was detected at the molecular mass of 969.1. However, when the phosphorylation reaction was carried out with ERK, the phosphorylated MBP3 peptide was also detected. The molecular mass of the phosphorylated MBP3 peptide was 1049.1 and was induced from $HPO_3$ ($M_r$ of $HPO_3$=80). Based on this result, we could confirm that the MBP substrate peptide was converted to phosphorylated peptide by ERK using MALDI–TOF-MS.

## 4. Conclusions

We obtained the optimal CE analysis conditions

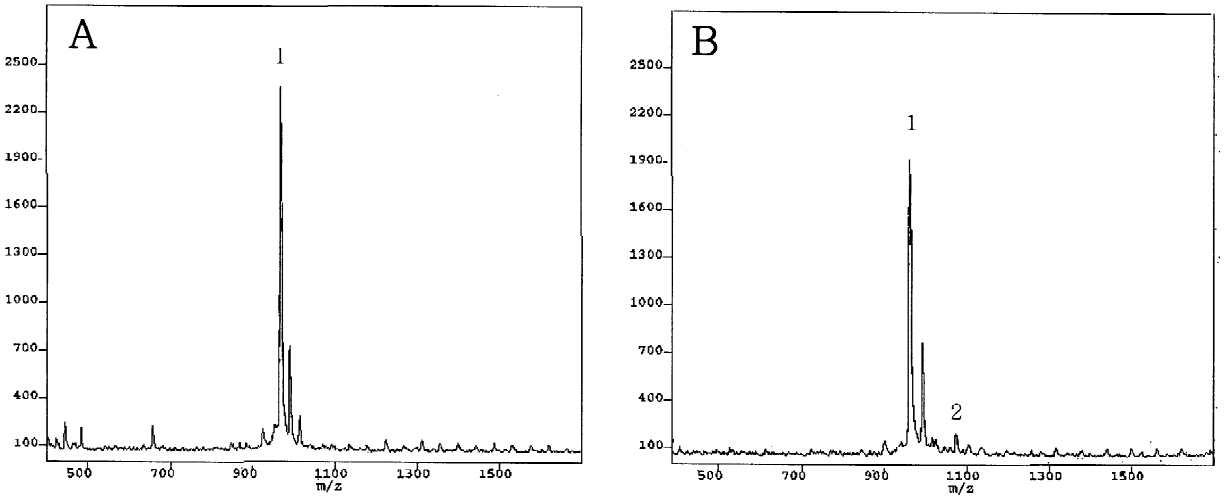


Fig. 5. MALDI–TOF mass spectrum of the phosphorylation reaction mixture without (A) and with 20 U/ml ERK (B). The phosphorylation reaction conditions and CE conditions are the same as in Fig. 2.

for the phosphorylation reaction of MBP substrate peptides by ERK. The analysis condition is 150 m*M* Tris–phosphate buffer containing 5% acetonitrile at pH 2.8, and 185-nm UV detection. Optimal reaction conditions for phosphorylation reaction by ERK using CE are as follows: reaction mixture prepared by mixing 5 μl of MBP peptides (0.1 mg/ml), 5 μl of ATP–$MgCl_2$ cocktail (500 μ*M* ATP and 75 m*M* $MgCl_2$ in kinase buffer) and 5 μl of kinase buffer (20 m*M* Tris–HCl buffer, pH 7.2, containing 25 m*M* β-glycerol phosphate, 5 m*M* EGTA, 1 m*M* DTT, 1 m*M* sodium orthovanadate). The reaction was initiated by adding ERK. Assays were performed at 30 °C for 30 min.

Using the obtained conditions, we compared the degree of phosphorylation reaction by characterization of MBP substrate peptides. In this result, we selected the MBP3 peptide as the effective substrate for the phosphorylation reaction by ERK. Under the obtained conditions, we identified the conversion of MBP3 peptide to phosphorylated MBP3 peptide using CE and confirmed this using MALDI–TOF-MS. Compared to conventional enzyme assays, this developed method is relatively rapid, easy, safety and has many advantages.

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