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# In vitro assay of the interaction between Rnc1 protein and Pmp1 mRNA by affinity capillary electrophoresis with a carboxylated capillary

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ABSTRACT

The interaction between Rnc1, an RNA interactive protein, and a Pmp1 mRNA was investigated by affinity capillary electrophoresis (ACE). Prior to the ACE experiments, the column performances of three capillaries (an untreated fused silica capillary, a polybrene–polyacrylic acid (PB–PAA) double layer coating capillary, and a carboxylated capillary with a covalent modification) were studied with model proteins including ribonuclease B (RNase B) and bovine serum albumin (BSA). Using an untreated fused silica and a PB–PAA double layer coating capillaries, both of the protein peaks were broad and tailing. However, using a carboxylated capillary, the protein peaks were sharp and symmetric, and migration times were repeatable (RSD < 0.4%). Further, the proteins in human serum also gave sharp peaks and its repeatability was kept at a high level by pre-treatment of a capillary inner wall with 1 M sodium chloride solution before each run. An Rnc1 protein was analyzed by ACE with background electrolytes containing various concentrations of Pmp1 sense mRNA using a carboxylated capillary. Increase in the concentration of the mRNA was found to delay the migration time of the protein. But the migration time of the protein was kept constant with increasing Pmp1 anti-sense mRNA instead of Pmp1 sense mRNA. A straight line ($r = 0.987$) was obtained by plotting 1/(migration time shift) *versus* 1/(Pmp1 sense mRNA concentration) and the association constant of Rnc1 protein with Pmp1 sense mRNA could be estimated to be $4.15 \times 10^6\ M^{-1}$. These results suggest that the association constants of proteins with mRNAs as ligands were easily determined by the proposed method.



## 1. Introduction

The mitogen-activated protein (MAP) kinases cascade is a crucial signaling pathway, since it regulates many physiological cell events such as cell growth, differentiation and death. Disturbance of MAP kinases signaling leads to cancer and other diseases. Recently, our group has reported that Pmk1 MAP kinase is phosphorylated and activated by Pek1 MAP kinase kinase, and dephosphorylated by Pmp1 phosphatase. Dephosphorylation of Pmk1 MAP kinase leads to decreased activity of Pmk1 [1,2]. We also discovered that activated Pmk1 phosphorylates Rnc1, which interacts with and stabilizes Pmp1 mRNA, resulting in elevated expression of Pmp1 protein. Therefore, the activity of Pmk1 is regulated by this negative-feed back loop through the phosphorylation of Rnc1. The most notable is that phosphorylation of the protein (Rnc1) affecting the interaction with the mRNA (Pmp1) controls activity of another important protein (Pmk1) [3]. In these chain reactions conversion of the Rnc1 protein to the phosphorylated form strongly influences the activity of Pmk1. A schema of the feed back mechanism is illustrated in Fig. 1. However, Rnc1 has many putative phosphorylation sites and the effects of the specific site(s) of phosphorylation remain obscure. Although we revealed that the negative-feed back loop using *in vivo* assay, it is rather difficult to compare the interactive strengths of the two proteins including the phosphorylated Rnc1 and the non-phosphorylated Rnc1 to the common RNA *in vivo*. Furthermore, it is also difficult to determine the interactions between the RNA and the proteins as association constants (*Ka*).

Several methods are used to study structures and/or functions of the proteins. These include electrophoretic techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [4–6], isoelectric focusing (IEF) [7,8], capillary zone electrophoresis (CZE) [9–11] and capillary gel electrophoresis (CGE) [12,13]. CZE and CGE are gaining in popularity because of their ease of operation and they have high resolution and sensitivity, which

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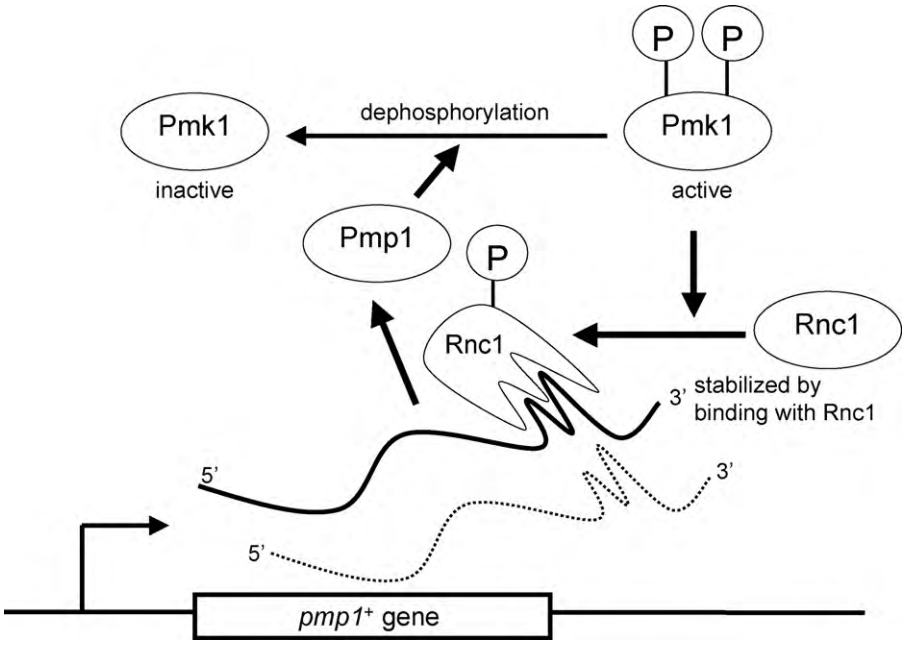


**Fig. 1.** Schematic of negative-feed back of Rnc1 by Pmk1 signaling.

means that the sample volume can be very reduced. Further, affinity capillary electrophoresis (ACE) which has high selectivity has been used by many researchers as a convenience tool for protein function studies, such as separation of analogs [14–16] and evaluation of affinity strength [17–19].

We also have previously used affinity mode of capillary electrophoresis (affinity capillary electrophoresis, ACE) to analyze differences of structures of proteins and ligands [20–25]. The association constants of proteins to carbohydrates [20–22], medicines [23], and glycoproteins [24,25] were measured based on migration time shifts with concentrations of interactants in the background electrolyte (BGE). Malonga et al. [26] also used ACE to determine the association constant of a transfer RNA with human serum albumin (HSA). On the other hand, non-specific adsorption of a protein to a capillary inner wall has adverse effects on ACE analysis. Tran et al. [27] used a fused silica capillary coated dynamically with poly(ethylene oxide) (PEO) to study the interaction between strongly basic proteins and DNA by ACE. We also modified the capillary inner wall with various compounds for ACE. A capillary dynamically coated with double layer of polybrene–polyacrylic acid (PB–PAA) was used to observe the interaction between a protein and a glycoprotein [25]. A capillary chemically coated with linear polyacrylamide was used for carbohydrate separation in a protein containing background electrolyte (BGE) [28,29]. In this work the interaction between the Rnc1 protein and the Pmp1 mRNA was investigated by ACE with a specially developed chemically modified fused silica capillary which had carboxyl groups on its inner wall to prevent adsorption of proteins.

## 2. Experiment

### 2.1. Chemicals

Bovine serum albumin (BSA) was obtained from Seikagaku Kogyo (Tokyo, Japan). Ribonuclease B (RNase B) was purchased from Sigma (St. Louis, MO). A pool sample of human serum from BIO Whittaker (Walkersville, MA, USA) was used as a protein mixture sample. All other chemicals were also of the highest grade commercially available. Glassware distilled water or autoclaved diethyl pyrocarbonate (DEPC) water after filtration with a membrane filter having 0.45 μm pore size was used for BGE and sample solutions. DEPC water was made by adding 2 mL of DEPC to 1 L of distilled water followed by autoclaving for 20 min at 121 °C, 2 atm. A stock solution of a phosphate buffer was prepared by adding a 50 mM disodium hydrogen phosphate aqueous solution to a 50 mM sodium dihydrogen phosphate solution until solutions with pH 6.8 was achieved with pH meter at room temperature (25 °C) and stored at 4 °C. This solution was used as a BGE for performance evaluation of a carboxylated capillary after filtration with membrane filter and degas. For ACE a stock solution of phosphate buffer was made with autoclaved DEPC water to inhibit RNase activity. This solution was used after addition of RNAs as ligands at appropriate concentrations.

### 2.2. Preparation of sense and anti-sense Pmp1 messenger RNA

Pmp1 cDNA was amplified by RT-PCR from the wild-type fission yeast total RNA and cloned into pBluescript vector (Stratagene, La Jolla, CA). The orientation of Pmp1 cDNA in the constructed vector was determined by restriction enzyme mapping. The sense Pmp1 mRNA was prepared by cutting the constructed vector by XhoI, followed by the transcription by T3 RNA polymerase. The anti-sense Pmp1 mRNA was prepared by NotI treatment and transcription by T7 RNA polymerase. The concentration of the mRNA was quantified by measuring the optical density at 260 nm.

### 2.3. Preparation of Rnc1 protein

For protein expression in *Escherichia coli* (*E. Coli*), the Rnc1 cDNA was amplified by RT-PCR from the wild-type fission yeast total RNA. Glutathion S-transferase (GST)-fusion proteins encoding Rnc1 were constructed using pGEX-4T3 (GE Healthcare, Waukesha, WI), expressed in *E. coli* XL1-blue, and purified using glutathione-Sepharose beads as previously described [2]. Rnc1contains 398 amino acids, with an approximate molecular mass of 43.3 kDa and an isoelectric point of 7.5.

### 2.4. Preparation of PB–PAA double layer coating capillary (for reference)

A PB–PAA double layer coating capillary of a physically adsorbed capillary was prepared according to our previous paper [25], briefly in below. A piece of capillary cut from a roll of fused silica (Polymicro Technologies, Phoenix, AZ, USA) having a detection window made by burning with thin flame was washed with 1 M sodium hydroxide for 10 min followed by distilled water for 5 min. And the washed capillary was coated by running 5% PB aqueous solution followed by 3% PAA for each 15 min with water rinsing between these solutions for 5 min. The capillary coated with double layer equilibrated by rinsing a BGE for 5 min before each sample introduction.

### 2.5. Capillary electrophoresis

#### 2.5.1. Capillary performance evaluation

Capillary electrophoresis was carried out with Applied Biosystems Inc. 270A capillary electrophoresis system to evaluate the basic performances of a carboxylated capillary. The apparatus was equipped with an auto sampler, an air circulating capillary oven and an UV detector. A FunCap-CE/Type C of a carboxylated capillary obtained from GL Science Inc. (Tokyo, Japan), bare fused silica as a reference and a PB–PAA double layer coating capillary as a reference were installed to the apparatus. Each capillary was of common inner diameter of 50 μm and common lengths of 72 cm as a total length and 50 cm as an effective length. Protein specimens of a RNase B as a basic protein and BSA as an acidic protein were dissolved in a BGE at a concentration of 1 mg/mL. A pool serum of human as a protein mixture sample was diluted 10-fold with a distilled water, because human serum contains sodium chloride at a high concentration of ca. 150 mM. Sample solutions were introduced by vacuum introduction method for 1.5 s from the anodic end of the capillary. A potential of +20 kV was applied, and UV absorption at 200 nm was monitored to detect. A capillary was conditioned by rinsing a BGE for 5 min before each run.

#### 2.5.2. Affinity capillary electrophoresis

Protein–RNA interactions were examined by capillary electrophoresis in the affinity mode using a Photal CAPI-3100 capillary electrophoresis system equipped with an auto sampler, an air circulating capillary oven and a multi-wavelength photodiode array UV detector. A carboxylated capillary (total length, 73.5 cm; effective length, 61 cm; inner diameter, 50 μm) was used. A protein sample of Rnc1 was prepared by dissolving in the BGE at 400 μg/mL. Stock solutions of BGE containing Pmp1 sense mRNA or Pmp1 anti-sense mRNA were prepared by dissolving to the BGE at 200 μg/mL, and the solutions were diluted with the same buffer to concentrations of 12.5, 50 and 100 μg/mL. The protein sample was analyzed in BGEs containing various concentrations of each mRNA. A potential of +20 kV was applied, and detection was carried out by monitoring UV absorption at 225 nm which is maximum absorption wavelength of GST-tag of the protein. Migration time shift of the protein depending on the mRNA concentration was observed to estimate the interaction between a protein and each mRNA.

## 3. Results and discussion

### 3.1. Capillary performance evaluation

#### 3.1.1. Analysis of protein specimens

To evaluate the capillary, RNase B and BSA were used as protein samples of a weakly basic protein and an acidic protein, respectively. When an untreated fused silica capillary was used as a separation column, RNase B and BSA were detected as relatively broad peaks at ca. 8 min and ca. 11 min, respectively (Fig. 2a). Electropherograms of protein specimens using a PB/PAA double-coated capillary are shown in Fig. 2b. RNase B was not detected at a concentration of 1 mg/mL or detected at much higher concentration of 10 mg/mL, but its peak was much broader than the peak obtained with the other capillaries. Polymers used for PB–PAA double layer coating capillary can make many loops near the capillary inner wall and the loops have a molecular sieving effect on velocities of positively charged samples migrating near the negatively charged capillary inner wall, resulting peak widths of positive charged large molecules broaden. However, when a carboxylated capillary was used the peaks of both proteins were sharp and symmetric, and both migration times of proteins were shorter than the other capillaries (Fig. 2c). Faster electroosmotic flow is caused by higher electric charge on the capillary wall, therefore expected number of negative charge on the carboxylated capillary is higher than the other two capillaries and RNase B of a weakly positive charged protein may strongly interact with the carboxylated capillary. However, in the presence of stronger negative charged anion like phosphate the interaction between protein and carboxylate can be inhibited, resultantly the carboxylated capillary gives good peak shapes as in Fig. 2c and high reproducibility described in below. In a case of untreated fused silica having silanol groups, even if ionic interaction was inhibited, amines of the basic proteins may interact with silanol groups stronger than carboxyl groups by hydrogen bonding. In these electropherograms, two peak tops of RNase B can be based on group separation of its glycoforms or its contamination of non-glycosylated form, and BSA separated into two peaks which can be based on its glycoforms or degrees of phosphorylation. Both proteins gave each single peak having one peak top in Fig. 2c, which means that heterogenic forms were not separated with a carboxylated capillary under the conditions used. For more accurate separation a longer capillary may help resolutions of these heterogenic proteins.

Reproducibility of migration time is an important factor in ACE because the *Ka* value is calculated from the migration time shift depending on the ligand concentration in a BGE. The repeatability of the migration time for each capillary was investigated under the same analytical conditions. The capillary was preconditioned by rinsing with a BGE for 5 min before each run for all capillaries. The average of migration times and repeatabilities, expressed as relative standard deviation (RSD) are summarized in Table 1. In the analysis of RNase B, RSD values of 6 times repetition with an untreated fused silica and a carboxylated capillary were 0.69% and 0.15%, respectively. RSDs of migration time of BSA with three capillaries were 1.17% (untreated fused silica), 0.33% (PB/PAA double-coated capillary) and 0.40% (carboxylated capillary). Although both carboxylated and PB/PAA double-coated capillaries gave excellent repeatability, the migration time of the proteins with a carboxylated capillary was much shorter than that of the proteins with a PB/PAA double-coated capillary. A carboxylated capillary allows reproducible and high speed analysis of both acidic and weakly basic proteins by capillary electrophoresis.

#### 3.1.2. Analysis of human serum

Human serum, a diverse mixture of proteins, carbohydrates, minerals, etc., and lipids was also analyzed to compare the three kinds of capillaries on separation performance and repeatability. Human serum was diluted 10-fold with distilled water and analyzed in nine successive runs by CE with the three capillaries (Fig. 3). Serum proteins were separated to mainly albumin and three globulin fractions under the same conditions used with all the three capillaries. An electropherogram of human serum in an untreated fused silica at the first run gave three peaks of globulins and a large peak of albumin, although each peak became broader and its migration time was increased by repetition (Fig. 3a), probably because

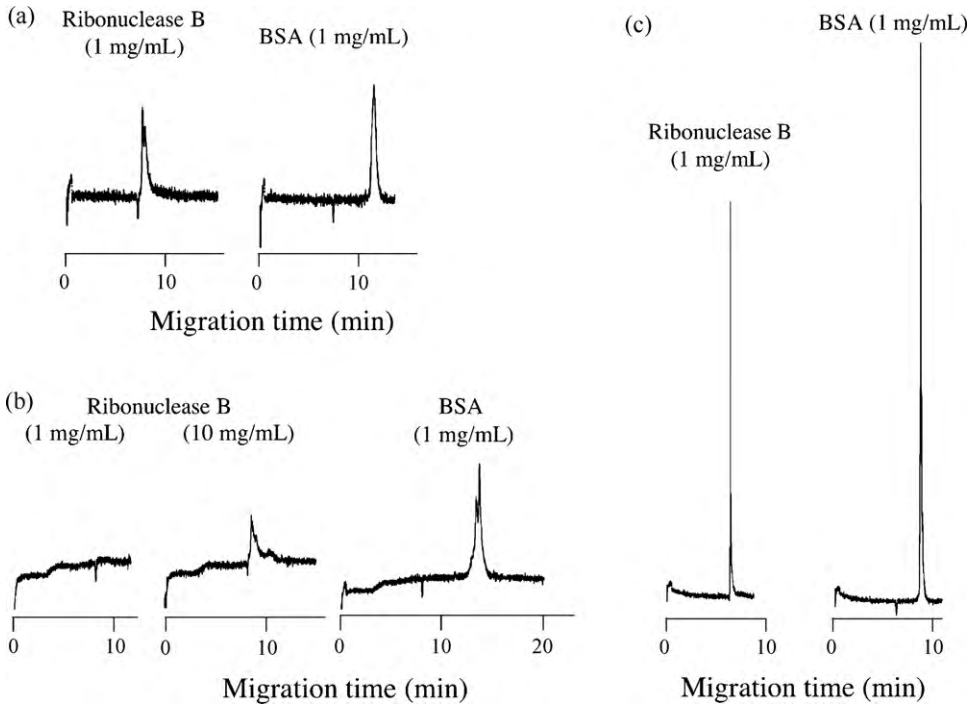


**Fig. 2.** Electropherograms of protein specimens. Capillary, untreated fused silica (a), PB/PAA double layer coated capillary (b), carboxylated capillary (c), each capillary have common total length, effective length and inner diameter of 72 cm, 50 cm and 50 μm, respectively; each capillary was rinsed with BGE for 5 min before sample introduction; BGE, 50 mM phosphate buffer pH 6.8, applied voltage, 20 kV; temperature, 30 °C; detection, UV absorption at 200 nm.

proteins and minerals including alkaline earth metals in the serum sample at high concentrations adsorbed to the capillary inner wall. A PB/PAA double-coated capillary gave excellent repeatability for serum analysis and sharp peaks of acidic proteins. However, the first peak which was detected around neutral in Fig. 3a was not detected as shown in Fig. 3b. With the carboxylated capillary, globulins were well separated into three fractions, and albumin was detected as a sharp peak. This capillary gave the optimal separation performance among the capillaries used. However, the migration time was gradually delayed by repetition of separation (Fig. 3c). This problem of repeatability was solved by rinsing with 1 M sodium chloride solution to wash the capillary inner wall before each run (Fig. 3d). As a result, a carboxylated capillary can be used for analysis of weakly basic to acidic proteins with high resolution and repeatability simply by treating of a capillary with 1 M sodium chloride solution.

### 3.2. *Affinity capillary electrophoresis*

In wild cells, Rnc1 protein can interact with Pmp1 mRNA to control MAP kinase activity as mentioned above. The interaction between Rnc1 protein and Pmp1 mRNA was determined by ACE. ACE analysis was carried out by analyzing a protein as a sample in BGEs containing mRNA as a ligand at various concentrations. Fig. 4a shows electropherograms of Rnc1 protein in the absence or presence of Pmp1 sense mRNA. The migration time of the protein increased with increasing concentration of mRNA in the BGE. However, Pmp1 anti-sense mRNA essentially did not affect the migration time of the protein (Fig. 4b). These results verify that the migration time shift by adding Pmp1 sense mRNA is based on a specific interaction. A ligand of mRNA added to the BGE in this system is strongly negatively charged polymer. Therefore mRNA moves by electrophoresis to the anodic end against the electroosmotic flow, and as a result it migrates slowly to the cathodic end in the presence of fast electroosmotic flow. Here a protein that has a low electric charge-to-size ratio migrates at about the same speed as the electroosmotic flow rate. However, when the protein forms a complex with the mRNA of a strongly negatively charged large molecule, the apparent migration velocity of the protein is decreased. The affinity constant can be estimated from the migration time shift according to our previous paper [22]. If the effects of viscosity, ion strength and/or other factors on the electroosmotic flow rate is/are terrible, the migration times can be corrected used mobility or relative mobility to the appropriate internal standard. In this case an internal standard must not be affect the interaction. If a mobility marker even weakly affected the interaction, and/or the interactants affect the migration rate of the marker, *Ka* should be estimated from migration times which were not corrected, but negative control must be tested. A plot of the reciprocal of the migration time shift $[1/(t - t_1)]$ against the reciprocal of the ligand concentration (1/[L]) gives a straight line, where $t_1$ and $t$ are the migration times in the absence or presence of ligand at the concentrations of [L], respectively. *Ka* can be estimated from the slope ($A$) and the $Y$-intercept ($B$) of the double reciprocal plot, and the migration time

**Table 1**
Repeatability of migration time for RNase B and BSA.

| Capillary | RNase B (weakly basic protein) | | BSA (acidic protein) | |
|---|---|---|---|---|
| | Migration time (min) | RSD (%, $n = 6$) | Migration time (min) | RSD (%, $n = 6$) |
| Untreated fused silica | 7.74 | 0.69 | 11.23 | 1.17 |
| PB/PAA double coated | – | – | 13.54 | 0.33 |
| Carboxylated | 6.25 | 0.15 | 8.63 | 0.40 |

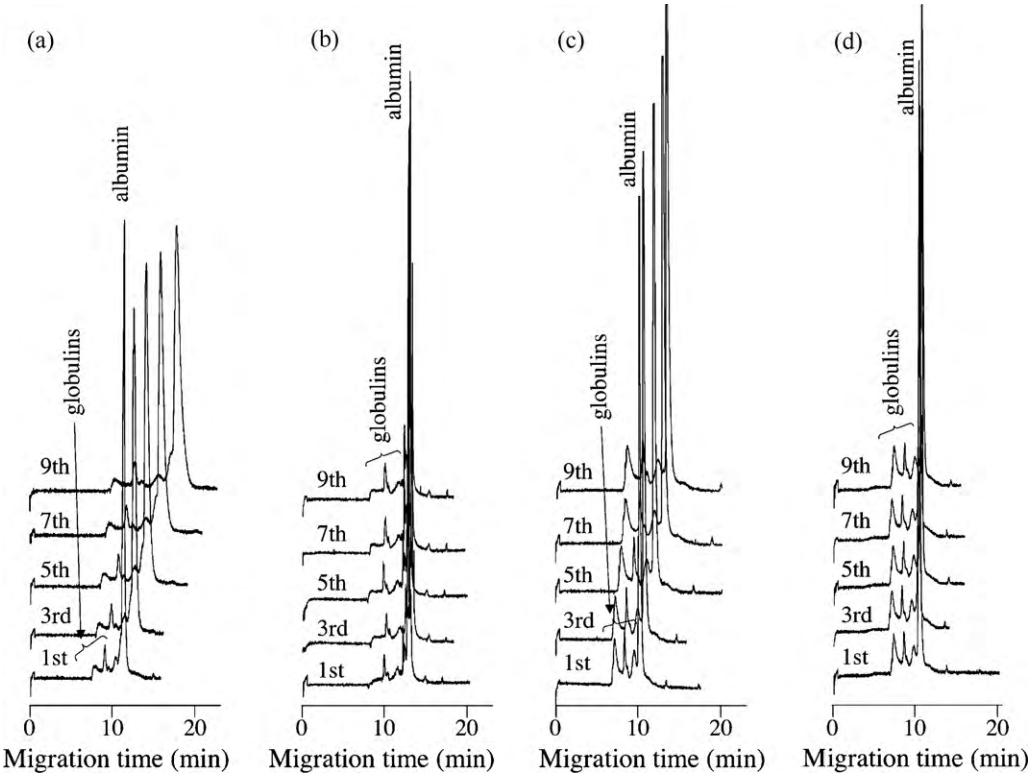


**Fig. 3.** Influence of capillary modification on separation of serum proteins. Capillary, untreated fused silica (a), PB/PAA double layer coated capillary (b), carboxylated capillary (c and d), capillaries were rinsed with BGE for 5 min before sample introduction (a–c), capillary was rinsed with 1 M NaCl for 1 min followed by BGE for 4 min (d); other conditions as in Fig. 2.

of the protein in the absence of ligand ($t_1$) by Eq. (1).

$$\mathrm{Ka} = (Bt_1 + 1)A^{-1}t_1^{-1}\ \mathrm{M}^{-1} \quad (1)$$

In the *in vitro* assay of the interaction between Rnc1 protein and Pmp1 sense mRNA, the double reciprocal plot gave a straight line with good linearity ($Y = 2.01 \times 10^{-7}X + 0.693$, $R = 0.987$). From the slope ($A$) and the $Y$-intercept ($B$) of the straight line, and the

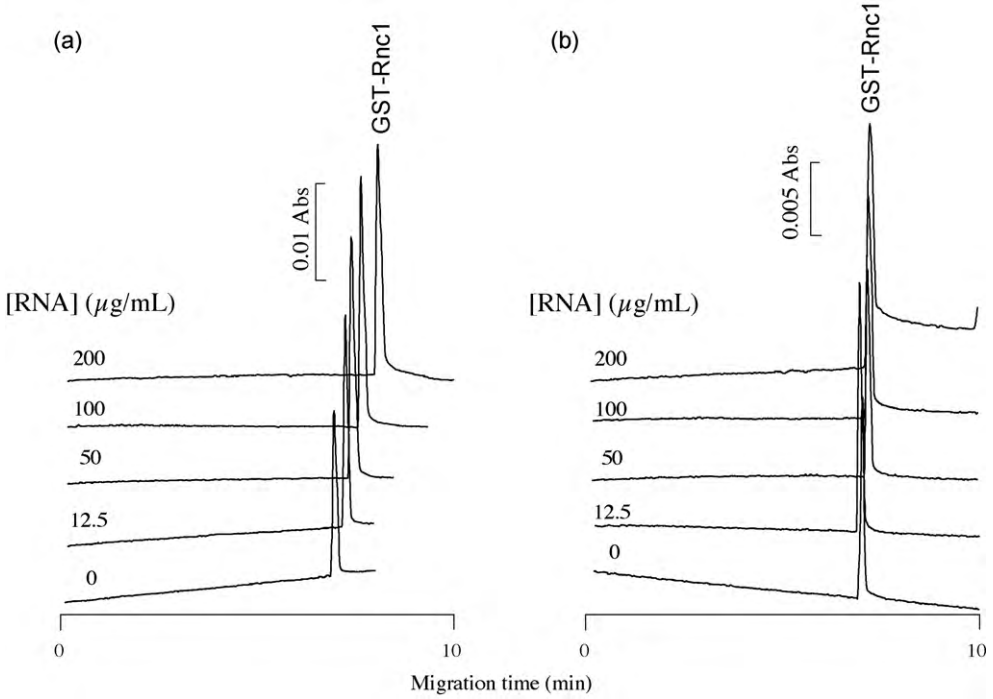


**Fig. 4.** Affinity capillary electrophoresis of Rnc1 protein in the presence of Pmp1 sense mRNA (a) or Pmp1 anti-sense mRNA (b). Capillary, carboxylated capillary (50 μm i.d., 73.5 cm, 61 cm); BGE, 50 mM phosphate buffer pH 6.8 containing various concentrations of Pmp1 sense mRNA (a) or Pmp1 anti-sense mRNA (b); applied voltage, 20 kV; temperature, 25 °C; detection, UV absorption at 225 nm.

migration time of Rnc1 protein in the absence of Pmp1 mRNA, *Ka* value was estimated to be $4.15 \times 10^6$ ($M^{-1}$). The order of this *Ka* value was comparable to the *Ka* between a peptide and transfer RNA measured by fluorescence spectroscopy [30].

## 4. Conclusions

A carboxylated capillary could be used for high performance protein analysis of wide range proteins with high repeatability, high resolution and short analysis time. Furthermore, this capillary could be used to analyze serum samples with high reproducibility if it is preconditioned with 1 M sodium chloride. To prevent adsorption of proteins, capillaries are usually chemically or dynamically coated with neutral polymers such as linear polyacrylamide, polyvinylalcohol, polyvinylpyrrolidone and polyethylene glycol. Although these coatings effectively prevent adsorption of proteins with strong positive or negative charges, simultaneous determination of acidic proteins and basic proteins is difficult using a neutral coated capillary. However, a strong advantage of CE separation with a carboxylated capillary is that allows simultaneous determination of acidic, neutral and weakly basic proteins because it not only prevents adsorption of proteins to its inner wall but also causes fast electroosmotic flow.

Using this special capillary, an association constant between a mRNA and an RNA binding protein could be determined from the migration time shift of the protein depending on the mRNA concentration in the BGE. The *in vitro* assay method developed in this study makes it possible to determine the affinity constant by simple operations of analyzing a protein in BGEs containing RNA at various concentrations by ACE. Only five runs in one assay were enough to determine the *Ka* value in this case.

This method can be used as a high throughput screening system to identify new candidates for cancer therapy or cancer markers. In the future we will use this method to screen proteins and nucleic acids which activate cancer or have anti-cancer activities. On the other hand, we measured the *Ka* of only a non-phosphorylated Rnc1 in this work, because Rnc1 protein used was produced by *E. coli*, resultantly the protein was not phosphorylated by post-translational modification. Therefore, we plan to synthesize phosphorylated Rnc1, and investigate the influence of phosphorylation on the interaction with mRNA by this system to elucidate one of a function in one of a carcinogenesis mechanism.

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