ABSTRACT
We report successful application of microchip phosphate-affinity electrophoresis (µPAE) to measurements of phosphorylation/dephosphorylation activities of kinases/phosphatases. We immobilized Phos-tag, a phosphate-specific affinity ligand, to the polymer matrix in a microchannel. Samples were prepared by reacting the substrate peptide with PKA or LAR PTPase. The immobilized Phos-tag selectively trapped the phosphorylated peptides, which were quantified with fluorescence. The limits of detection were 1.2% (PKA) and 1.5% (LAR PTPase) product peptides in the total peptides. We also successfully detected the kinase activities in cell lysates. The µPAE provides rapid and sensitive way to measure activities of kinases and phosphatases.

KEYWORDS: Affinity Electrophoresis, Microchip, Kinase, Phosphatase, Phos-tag

INTRODUCTION
Intracellular signaling networks are known to be constructed on the basis of the subtle balance between phosphorylation and dephosphorylation. Two key classes of enzymes, kinases and phosphatases, are involved in catalyzing the opposing activities of phosphorylation and dephosphorylation of proteins[1]. Emphasizing the importance of protein phosphorylation is the existence of more than 500 protein kinases encoded within the human genome with more than 200 of these enzymes implicated in human diseases. Phosphatases act in opposition to kinases, and are integral to many signal transduction pathways. The activities of many protein kinases are regulated by phosphorylation [2]. The phosphorylated kinases thus comprise an important class of substrates for protein phosphatases. Therefore, to investigate the regulatory mechanisms of signal transduction by protein phosphorylation, it is important to develop techniques for detecting and analyzing both protein kinases and protein phosphatases.

Using Phos-tag Acrylamide and a poly(dimethylsiloxane) (PDMS)-glass microchip, we demonstrated microchip phosphate-affinity electrophoresis (µPAE) [3]. For the µPAE, we loaded a Phos-tag–poly(dimethylacrylamide) (PDMA) conjugate and a peptide sample solution into the microchip using our original, autonomous solution filling technique [4]. The sample solution contained a substrate peptide of a tyrosine kinase, c-Src, and its phosphorylated product. The latter was trapped by Phos-tag, while the former migrated. As a result, the two peptides were baseline separated in 10 s.

Compared with our first report on µPAE [3], this paper describes three advances. (1) Application to a serine/threonine kinase, PKA, is demonstrated, while only a tyrosine kinase (Src) was previously measured. (2) Application to a phosphatase (leukocyte antigen-related protein tyrosine phosphatase; LAR PTPase) is demonstrated, while µPAE was previously limited to kinases. (3) Application to more realistic samples (cell lysates) than those of our previous report (purified recombinant enzymes) is described.

EXPERIMENTAL
Table 1 lists the sequences of the peptides used in this work. The peptides were fluorescently labeled at their N termini. For standard samples of the PKA assay, P1 and P2 were mixed in a 50 mM Tris–HCl buffer (pH 7.5) at various molar ratios while keeping the total peptide concentration at 10 µM. Similarly, P3 and P4 were mixed to prepare standard samples of the LAR PTPase assay.

<p>| Table 1 Sequences of the peptides used in this work |</p>
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Sequence</th>
<th>Description</th>
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<tbody>
<tr>
<td>P1</td>
<td>FAM-EEELRRASLG</td>
<td>PKA substrate</td>
</tr>
<tr>
<td>P2</td>
<td>FAM-EEELRR[pS]LG</td>
<td>PKA product</td>
</tr>
<tr>
<td>P3</td>
<td>FAM-DADE[pY]LIPQQG</td>
<td>LAR substrate</td>
</tr>
<tr>
<td>P4</td>
<td>FAM-DADEYLIPOQQG</td>
<td>LAR product</td>
</tr>
<tr>
<td>P5</td>
<td>FITC-EEEIYGEFD</td>
<td>Src substrate</td>
</tr>
</tbody>
</table>

For preparation of the enzymatically reacted samples, the PKA reaction mixture was composed of 5–20 U mL⁻¹ PKA, 10 µM P1, 100 µM ATP, and 10 mM MgCl₂ in 50 µL of 15 mM Tris–HCl buffer (pH 7.5); The PTPase reaction mixture was composed of 0.1–0.4 U mL⁻¹ LAR PTPase, 10 µM P3 in 15 mM Tris–HCl buffer (pH 7.4).
Activity of Src in cell lysates was measured with μPAE as follows. MCF-7 cells were cultured, and then stimulated with nerve growth factor (NGF; Src activator) or staurosporine (STP; Src inhibitor). The cells were disrupted by ultrasonication, and the membrane fraction was extracted with 0.2% Triton X-100. The extract was reacted with P5 (Table 1), and then analyzed by μPAE.

We synthesized two Phos-tag–poly(N,N-dimethylacrylamide) (Phos-Tag–PDMA) conjugates with different Phos-tag concentrations: 0.10 mM and 1.0 mM. The synthesis protocol, fabrication of the PDMS-glass microchip and procedure of the electrophoresis were described elsewhere [3].

RESULTS AND DISCUSSION

The principle of μPAE is depicted in Figure 1a. In advance, the fluorescently labeled substrate peptide is reacted with the kinase or phosphatase. During the microchip electrophoresis, only phosphorylated peptide is trapped by Phos-tag (phosphate-binding ligand [5]), while non-phosphorylated peptide migrates. As a result, the two peptides are separated (Figure 1b) and quantified with the peak areas (Figure 1c). The kinase (or phosphatase) activity can be estimated by the amount of phosphorylated (or non-phosphorylated) peptide.

![Fig. 1](image)

(a) Schematic of the μPAE separation at the entrance of the Phos-tag-PDMA region. (b) Consecutive fluorescence images of the μPAE separation of an equimolar mixture of P1 and P2. (c) Fluorescence intensity profile plot of (b), 15 s, with the notation of .

Figure 2 shows the results of experiments regarding PKA. As shown in Figure 2a, the % phosphorylation at μPAE time of 15 s was perfectly proportional to the molar fraction of P2. Next, P1 was reacted with PKA, and the product mixture was analyzed with μPAE. The results are shown in Figure 2b. As expected, the % phosphorylation was raised with the PKA concentration and the reaction time. The same samples were also analyzed by MALDI-TOF MS, resulting in good agreement (data not shown). The limits of detection was 1.2% product peptides in the total peptide.

![Fig. 2](image)

(a) % Phosphorylation plotted against the molar fraction the PKA standard sample, P1 and P2. (b) % Phosphorylation plotted against the PKA reaction time with different PKA concentrations. % Phosphorylation was defined as $100 \times \frac{A_{\text{trap}}}{A_{\text{trap}} + A_{\text{mig}}}$.
Figure 3 shows the results of experiments regarding LAR PTPase. They are similar to those of PKA; standard samples consisting of P3 and P4 (Figure 3a) and enzymatic reaction products from P3 (Figure 3b). There are two differences from Figure 2. (1) The y-axes represent %dephosphorylation, which is equal to (100-%phosphorylation) in Figure 2. (2) The µPAE time was 10 s. This faster separation was possible because of the stronger binding of Phos-tag to phosphor-tyrosine than phosphor-serine. For the LAR PTPase, the limits of detection was 1.5% product peptides in the total peptide.

![Graph](image)

**Fig. 3** (a) % Dephosphorylation plotted against the molar fraction of the LAR PTPase standard sample, P3 and P4. (b) % Dephosphorylation plotted against the LAR PTPase reaction time with different LAR PTPase concentrations. % dephosphorylation was defined as \(100 \times \frac{A_{\text{mig}}}{A_{\text{trap}} + A_{\text{mig}}}\).

Figure 4 show the result of cell assay. MCF-7 cells were stimulated with Src activator nerve growth factor (NGF) and inhibitor staurosporine (STP), and then analyzed by µPAE. As shown in Figure 4, we successfully observed the Src activities enhanced by NGF and inhibited by STP.

CONCLUSION
We have demonstrated successful applications of the µPAE method to activity measurements of a serine/threonine kinase (PKA), and a tyrosine phosphatase (LAR PTPase). In both cases, the assay performances were as good as that of a tyrosine kinase c-Src that we previously reported [3]. We also applied successfully to activity monitoring of protein kinase in cell lysates.

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REFERENCES

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