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Research Article

Feasibility studies on a protein kinase assay when using radioisotope detection technique for developing a protein biochip

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Abstract: Microarrays have recently become a precious research tool for proteomics and clinical investigation. Their applications to the diagnosis of a disease have emerged as a significant promise for medical advances. In this study, we report on an efficient strategy for the detection of phosphorylation of a substrate catalyzed by kinase, using the radioisotope (RI) detection technique for a protein biochip. This technique does not employ the use of the blocking step which is commonly used in conventional methods to prevent non-specific binding. It was found that the usage of a RI detection technique has the advantages of being highly sensitive and time saving when compared with other conventional methods. The results can be applied when using RI detection technique to develop biochips to determine the activity of a protein kinase. Further, it can be a useful tool for a high throughput screening and for studying protein–protein interactions. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: radioisotope detection; phosphorylation detection; biomolecule interaction; biochip; microarray

Introduction

In recent years, we have begun to recognize the strengths and limitations of the genomics revolution, but it is difficult to keep up with proteomics which promises an even more radical transformation of biological and medical research. Microarrays function as a powerful tool for proteomics and clinical assays. They can be used to screen thousands of binding events in a parallel and high throughput fashion and are of major importance in the detection of a disease and drug discovery.[1] A protein microarray is a piece of glass slide on which various proteins are impregnated and it is used to identify protein–protein interactions. For example, it is used to identify the substrates of enzymes like protein kinases (PKAs) or the targets of biologically active small molecules. The signal detection can be visualized by autoradiography and quantified by a densitometer.[2,3] Current detection strategies for protein microarrays are broadly divided into label-free methods and labeled-probe methods. Labeled-probe methods are those that employ the use of fluorescence, chemiluminescence, electrochemiluminescence and radioactivity detection.[1,4] The use of radioisotopes (RIs) is traditionally considered to be one of the most sensitive labeling procedures. At present this technique is being developed to detect active proteins and to allow for a precise quantification of the amount of protein assayed.

Phosphorylation is a ubiquitous cellular regulatory mechanism found in all cells of the body. It occurs through the addition of a phosphate group to an amino acid residue via transfer of a terminal phosphate from an adenosine triphosphate (ATP) molecule catalyzed by enzymes, namely, kinases or phosphatases.[5–7] These kinases and phosphatases are of great interest to researchers involved in drug discovery and development, because of their role in a wide variety of diseases. Protein microarrays offer an ideal system for a rapid and parallel identification of the substrates for PKA. To investigate this application, we chose cAMP-dependent PKA and kemptide (a peptide substrate of PKA, LRRASLG) for phosphorylation. PKA is a ubiquitous serine protein kinase present in a variety of tissues.[8]

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The intracellular cAMP level regulates cellular responses by altering the interaction between the catalytic (C) and regulatory (R) subunits of PKA.[9] In the present study, we describe a suitable strategy for the detection of a phosphorylation of a kemptide catalyzed by PKA by using RI detection technique when developing a protein biochip. The use of RIs in protein microarray is widely preferred rather than other detection methods because it is highly sensitive, thus facilitating the detection of even minor quantities of protein.

Results and discussion

A novel strategy for a protein–peptide fusion protein interaction with an enzyme due to a steric accessibility was developed by Lee *et al.*[10–11] This strategy was enhanced by a peptide–enzyme interaction by using a fluorescence detection on a microarray. In this study, we investigated the feasibility of applying RI detection technique to a PKA assay for the development of a protein biochip. The substrate *Escherichia coli* malic enzyme–kemptide fusion protein was expressed as a soluble protein from a recombinant plasmid pTLMK3 in *E. coli* and purified by an affinity column. Protein samples obtained from each purification step were evaluated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The final step yielded a protein of a molecular weight of 65 kDa, which was identical with the estimated molecular weight (data not shown).

Phosphorylation of a cAMP-dependent PKA and an *E. coli* malic enzyme–kemptide fusion protein using [γ-^{32}P]ATP is illustrated in Figure 1. Purified *E. coli* malic enzyme–kemptide fusion protein or kemptide was spotted and immobilized on aldehyde-terminated glass slides and each slide was incubated in a reaction mixture that contained a kinase buffer supplemented with the ATP mixture (100 μM ATP, 10 μCi/μL [γ-^{32}P]ATP) and ten units of PKA. One unit of PKA is the amount of enzyme required to incorporate 1 pmol of phosphate into casein in 1 min. The substrate on a slide was washed and detected by using X-ray film and phosphoimager analysis. Specific signals of the kinase reaction were detected only for the fusion protein and peptide, but not for the bovine serum albumin (BSA) which was used as a negative control (Figure 2). Moreover, the fusion protein showed stronger signals than the peptide due to its steric accessibility.

We then examined the signal intensity with respect to varying concentrations of [γ-^{32}P]ATP in the kinase assay. Purified fusion protein was immobilized and incubated with PKA in the presence of [γ-^{32}P]ATP. The reaction mixture consisted of a kinase buffer supplemented with 100 μM ATP, various concentration of [γ-^{32}P]ATP ranging from 0.1, 0.2, 0.4 and 0.6 μCi/μL and ten units of PKA. The signal intensity increased up to 0.4 μCi/μL and thereafter reached saturation (Figure 3). It is shown that the kinase activity could be determined even with less than 0.1 μCi/μL of [γ-^{32}P]ATP. We also measured the signal intensity of the kinase assay with different concentrations of PKA. The reaction mixture consisted of a kinase buffer supplemented with the ATP mixture (100 μM ATP, 10 μCi/μL [γ-^{32}P]ATP) and various concentrations of PKA (0.1, 1, 10, 20, 30, 40 and 50 U/mL were also added). It was observed that the signal intensity was

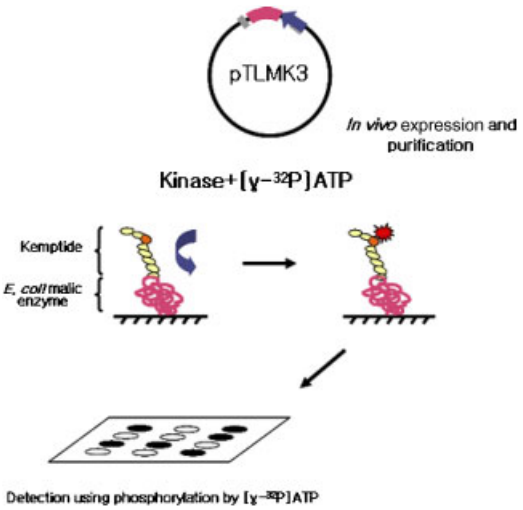

Figure 1 Scheme of a recombinant protein substrate for the kinase assay on a radioactive isotope. The gene, protein, peptide and transferred [^{32}P]phosphate shown in different colors are as follows: pink, *E. coli* malic enzyme; yellow, kemptide; red, [^{32}P]phosphate. This figure is available in color online at www.interscience.wiley.com/journal/jlcr

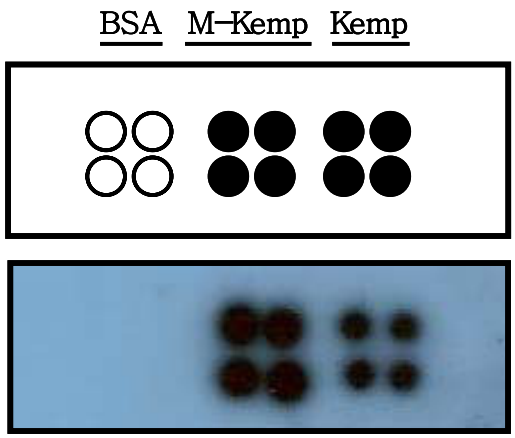

Figure 2 Detecting the substrate of PKA with [γ-^{32}P]ATP on a glass slide. BSA, bovine serum albumin; M-Kemp, *E. coli* malic enzyme–kemptide fusion protein; Kemp, kemptide.

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detected from 0.1 U/mL of PKA which gradually increased up to 10 U/mL of the enzyme concentration. There was no further increase in the signal intensity, possibly due to saturation at 20 U/mL of the enzyme concentration (Figure 4). These results confirm that the kinase activity could be detected even with a minimum concentration of the enzyme (0.1 U/mL). The signal intensity of the kinase activity at different time periods of the reaction was also determined. The reaction mixture contained 100 μM of ATP, 10 μCi/μL of [γ-^{32}P]ATP and ten units of PKA and it was incubated for different time periods ranging from 0 to 60 min at 37°C. Figure 5 shows that there was no signal intensity up to 20 min of the reaction time, and then it was clearly perceived after 30 min. The signal intensity was nearly same from 30 to 60 min. These results indicate the advantage of RI detection technique in saving the reaction time.

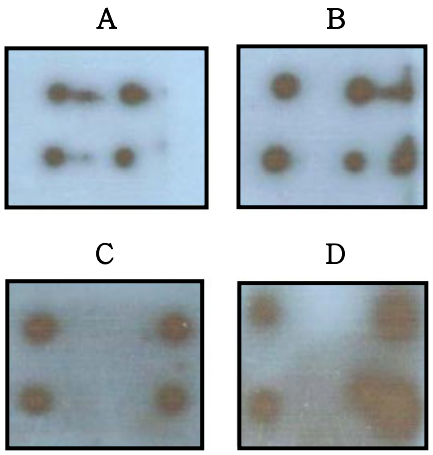

Figure 3 Effect of [γ-^{32}P]ATP concentration on the kinase assay. A, 0.1 μCi/μL; B, 0.2 μCi/μL; C, 0.4 μCi/μL; D, 0.6 μCi/μL [γ-^{32}P]ATP.

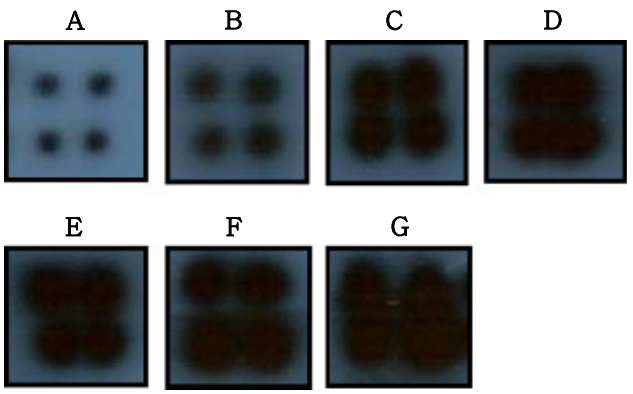

Figure 4 Effect of PKA concentration on the kinase assay. A, 0.1 U/mL; B, 1 U/mL; C, 10 U/mL; D, 20 U/mL; E, 30 U/mL; F, 40 U/mL; G, 50 U/mL cAMP-dependent protein kinase.

BSA is normally used as a blocking agent during immunostaining using a primary antibody to prevent a non-specific binding of proteins to tissue sections or cytological samples.[4] However, the major problem associated with the use of BSA is that they cause immobilized peptide to be hardly accessible to macromolecules such as enzymes and antibodies.[12] Hence, we were interested in establishing a more efficient blocking agent for a biochip. Purified *E. coli* malic enzyme–kemptide fusion protein or kemptide was spotted and immobilized on aldehyde-terminated glass slides. Each slide was then incubated with PKA in the presence of [γ-^{32}P]ATP. Prior to incubation, the slides were treated with none, 1% BSA, 1% glycine and 10% glycerol, respectively. The results indicate that all the samples presented a very clear signal intensity as shown in Figure 6. Hence, it can be confirmed that, there is no need for a blocking step on a biochip. The results of the present investigation provide certain advantages. First, the method is very simple and highly sensitive than other conventional methods, as it makes use of RI detection technique. Secondly, this method considerably reduces the experiment time, as the use of blocking agent can be avoided. Third, they facilitate immobilization and identification of small molecules (kemptide) on a biochip which is not possible with other methods.

In a conventional enzyme-linked immunosorbent assay (ELISA), interaction between a kinase and a peptide is practically not feasible since its small size causes the peptide to get buried in a bigger BSA.[13] Moreover, it is difficult to detect a phosphorylated peptide with fluorescence-labeled antibodies. Minimal

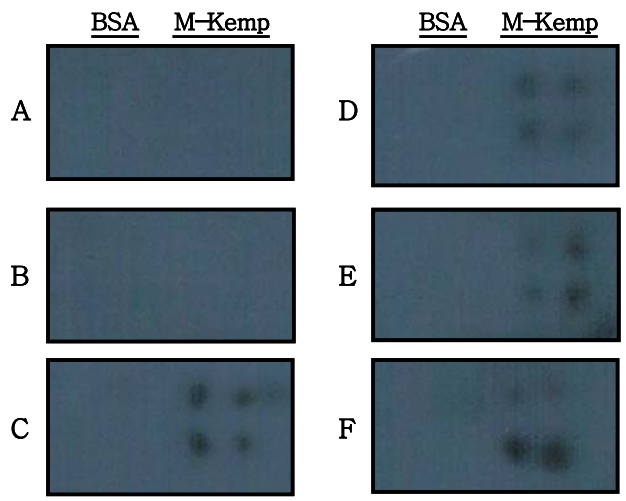

Figure 5 Signal intensity according to the reaction time of an enzyme. BSA, bovine serum albumin; M-Kemp, *E. coli* malic enzyme–kemptide fusion protein A, 10 min; B, 20 min; C, 30 min; D, 40 min; E, 50 min; F, 60 min.

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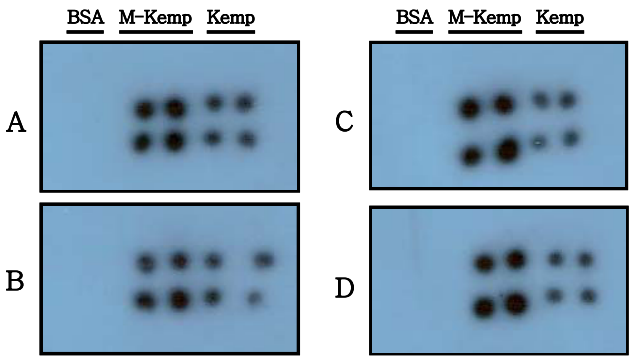

Figure 6 Efficient blocking agent on a chip. A, none; B, 1% BSA; C, 1% glycine; D, 10% glycerol. BSA, bovine serum albumin; M-Kemp, *E. coli* malic enzyme–kemptide fusion protein; Kemp, kemptide.

non-specific binding of biomolecules on a biochip is crucial for high-quality microarray experiments. These problems can be overcome by using RI detection technique since it reduces the overall detection time and also it is highly sensitive thus facilitating easy detection. Further, it does not make use of blocking agents that offers minimum non-specific binding, thereby facilitating identification of smaller molecules which is practically not feasible with other conventional methods.

Experimental

Chemicals, peptides and plasmid

PKA assay kit (the catalytic subunit of PKA and its substrate kemptide) was purchased Promega (Madison, WI). The aldehyde-terminated slides were purchased from Nuricell Inc. (Seoul, Korea). [γ-^{32}P]ATP was purchased from GE Healthcare Life Science (Buckinghamshire, UK). Unless specified, all other reagents were obtained from Sigma (St. Louis, MO).

Strains and plasmids

Plasmid pTLMK3 was a kind gift from Korea Advanced Institute of Science and Technology (Daejeon, Korea). DNA encoding kemptide peptide fused to the *E. coli sfcA* gene (encoding malic enzyme) was obtained by overlap PCR amplification using plasmid pEDOb5[11] carrying the genomic DNA from *E. coli* W3110 as a template.

Expression and purification of the recombinant fusion proteins

For preparation of *E. coli* malic enzyme–kemptide fusion protein from the cells, the organism was grown in LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) containing final concentration 50 μg/mL ampicilline at 37°C and was expressed in 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). The cells were harvested by centrifugation and disrupted by sonication in ice-cold PBS buffer (200 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, 1 mM Na_2HPO_4, pH 7.5). The crude recombinant fusion protein with His-tag at their N-terminal was purified by a HiTrap chelating HP column using a gradient from 0 to 500 mM imidazole in 20 mM sodium phosphate, pH 7.4 containing 500 mM NaCl. The eluted recombinant fusion protein was dialyzed against PBS buffer, pH 7.5. This recombinant fusion protein can be directly spotted and immobilized on the slide glass for any further purification process. The protein compositions of envelope proteins were determined by SDS-PAGE. Protein concentration was measured by the method of Bradford (Bio-Rad protein assay, Bio-Rad) using BSA as a standard.[14]

Determination of PKA activity

PKA activity was measured using SignaTECT PKA System (Promega) according to the manufacturer's recommendations with some modification. The *E. coli* malic enzyme–kemptide fusion protein and biotinylated kemptide diluted to 0.1 mg/ml and 1.25 μg/ml with PBS buffer containing 10% glycerol were spotted on sticker chip (the aldehyde-terminated slides). For immobilization of substrate, prepared slides were incubated in a humid chamber for 1 h at 30°C. In the case of necessity, the slides were dropped into PBS buffer containing 1% (w/v) BSA for blocking. The slides were washed with PBS buffer and kinase buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.5) in sequence. The slides were incubated with reaction mixture for 1 h at 37°C. The reaction mixture was composed of the kinase buffers supplemented with ATP mixture (100 μM ATP, 10 μCi/μL [γ-^{32}P]ATP) and ten units of cAMP-dependent PKA. After reaction, the slides were washed three times for 30 s. After the slides were exposed to an X-ray film (Fuji) or imaging plate, they were developed or analyzed by bioimage analyzer (Fuji photo Film, Tokyo).

Conclusion

In this study, we have performed a feasibility study on using [γ-^{32}P]ATP for determining the activity of a PKA assay on a glass chip. The results are found to be promising and can be applied when using RI detection technique for a biochip to determine an enzyme activity. It can also be a useful tool for a high throughput

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screening (HTS). The use of a RI detection technique for a protein biochip offers certain advantages that cannot be obtained with other conventional methods. Further, this technique is highly sensitive and since it does not make use of blocking agents, the probability of obtaining a false positive result is negligible.

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