ORGANIC LETTERS

2005
Vol. 7, No. 25
5565−5568

On-Bead Fluorescence Assay for Serine/Threonine Kinases

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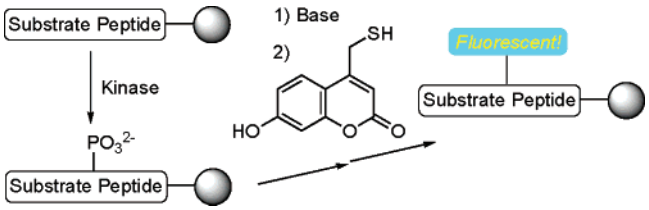
Received September 3, 2005

ABSTRACT



A novel fluorescence-based assay for serine/threonine kinases is described. Base-mediated β-elimination of the phosphate moiety and the Michael addition of a thiol-containing fluorescent molecule allows convenient and efficient detection of the enzyme activity. This approach may be broadly applicable to various serine/threonine kinases.

Protein kinases catalyze phosphoryl group transfer from adenosine triphosphate (ATP) to serine, threonine, and/or tyrosine residues of target proteins. Protein phosphorylation is one of the most common posttranslational modifications and plays an important role in intracellular signal transduction. Protein kinases ultimately regulate many aspects of cellular function, including proliferation, differentiation, the cell cycle, metabolism, transcription, and apoptosis.[1] Protein kinase genes form one of the largest gene families in eukaryotes; in humans they are estimated to account for 1.5−2.5% of all genes.[2] A key feature of protein kinases is substrate specificity, which is mainly determined by the primary sequence around the phosphorylation site of the target proteins. As a result, great efforts have been made to identify the potential kinase substrates, including combinatorial synthesis of peptide libraries with radioisotope-based or antibody-based detection.[3] Protein kinases are also becoming attractive targets for drug discovery, since many of them are associated with a wide variety of diseases, such as cancers, cardiovascular disease, and inflammation.[4] There is a great impetus to develop small-molecular inhibitors specific to a particular kinase, for both therapeutic reasons[5] and enzyme functional analysis.[6]

Conventional assay for protein kinase activity is based on the transfer of ^{32}P from γ-^{32}P-ATP to the target peptides or proteins.[7] This method is widely used, but radiolabel represents a potential risk to human health and to the environment. In addition, the long exposure time needed for sensitive detection of ^{32}P does not lend itself to high-throughput applications. The use of antibodies directed against phosphorylated residues is also popular,[8] but the specificity of the antibodies is problematic in some cases. Several fluorescent probes have been developed recently,[9−11] such as Zn^{2+}-based artificial phosphate receptors[9] and peptide

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10.1021/ol052125k CCC: $30.25
Published on Web 11/10/2005

substrate bearing a phosphate sensor.[10] These probes are elegantly designed, but their affinity and sensitivity could be affected by the amino acid sequence of the target phosphopeptide.[9b] Therefore, a convenient assay method would become a powerful tool for kinase research. Here we report the novel fluorescence-based assay for serine/threonine kinases, which is applicable to high-throughput screening.

Our approach is summarized in Figure 1. The key reactions

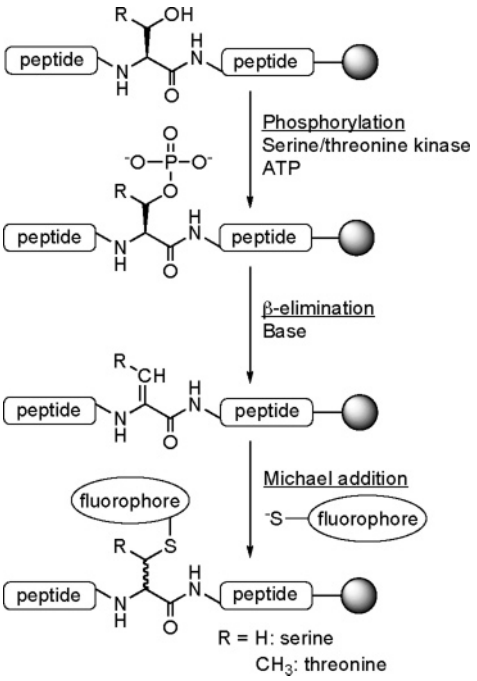
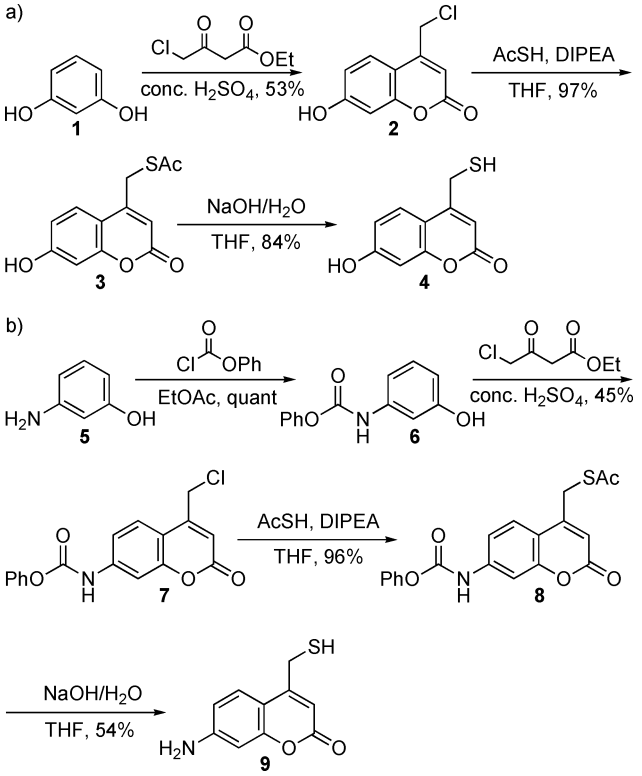


Figure 1. Summary of the assay.

are base-mediated β-elimination of the phosphate moiety and the Michael addition of a thiol-containing fluorescent molecule, which enable the selective transformation of phosphoserine and phosphothreonine residues into fluorescent derivatives. Although several researchers have used similar chemistry to enrich and quantify phosphopeptides, identification of the sites of phosphorylation typically relied on tandem mass spectrometry to sequence individual peptides.[12] MS spectrometry is not necessarily suitable for quantification and is difficult to apply to high-throughput screening. We adopted solid-supported peptide substrate and fluorescence detection to achieve a rapid, safe, and sensitive assay.

The thiol-containing fluorescent molecules were designed and synthesized according to Scheme 1. We selected

Scheme 1 Synthesis of Compounds **4** (a) and **9** (b)



coumarins as the fluorophore because of their high fluorescence quantum yield and low hydrophobicity. Their relatively hydrophilic nature is expected to reduce nonspecific interaction with the solid support; such an interaction would potentially lower the reliability. We designed the hydroxycoumarin derivative **4** and the aminocoumarin derivative **9**. To examine their reactivity, the reaction was performed with methyl vinyl ketone as a model Michael acceptor under basic conditions.

Both **4** and **9** gave the desired compounds **10** and **11**, respectively. As shown in Figure 2, the fluorescence intensity of **10** was much stronger than that of **4**, suggesting that the fluorescence from nonreacted dye is weaker than that from the reacted dye. Compound **4** was found to be fairly stable in the buffer at pH 9.4; almost no disulfide product was detected by HPLC (see Supporting Information). Similar fluorescence spectra were obtained with aminocoumarin derivatives **9** and **11** (see Supporting Information). This characteristic will reduce the background fluorescence from the dye nonspecifically bound to the solid support. The fluorescence properties of coumarin derivatives are known to be influenced by the degree of intramolecular charge transfer (ICT)[13] and/or photoinduced electron transfer (PET)[14a] from the substituents to the coumarin ring. Although the

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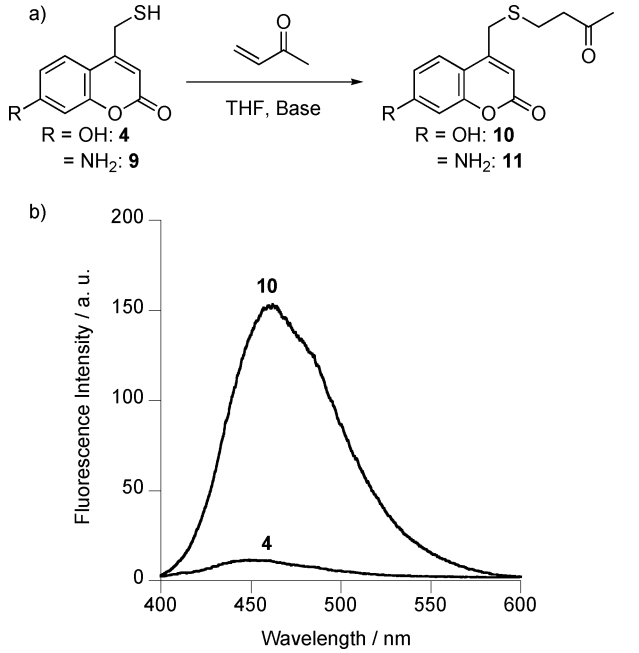

Figure 2. (a) Reaction of **4** and **9** with methyl vinyl ketone. (b) Emission spectra (ex 360 nm) of **4** and **10** (5 μM) in 100 mM glycine/NaOH buffer (pH 9.4) containing 0.1% DMSO.

reason for the weak fluorescnece of thiol-containing coumarins is not entirely clear, the PET mechanism is supposed to be appropriate. The formation of **10** from **4** led to little change in the absorbance maxima but increased the fluorescence intensity, which is a characteristic feature of PET-type fluorescent probes.[14]

We first optimized the conditions of β-elimination and Michael addition in solution. As a peptide substrate, we chose Kemptide (Ac-LRRASLG-NH_2), which is the well-known target sequence of cAMP-dependent protein kinase (PKA).[15] Both Kemptide and phosphorylated Kemptide (Ac-LRRA-pSLG-NH_2) were synthesized with standard Fmoc solid-phase methods. The reactions were monitored by HPLC (see Supporting Information), and conditions were found under which both β-elimination and Michael addition proceeded. Although $Ba(OH)_2$ was reported as a good reagent for β-elimination,[16] NaOH worked better in our case. It was confirmed that **4** and **9** were selectively introduced into the phosphorylated Kemptide but not into Kemptide itself. The products were characterized by MALDI-MS (see Supporting Information).

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To examine the conditions for on-bead assay, Kemptide and phosphorylated Kemptide were synthesized on TentaGel S NH_2 resin; the peptides were deprotected but remained bound on the resin under the normal deprotection/cleavage conditions. It was found that the conditions optimized for the solution-phase reaction were applicable with slight modification. Dye **4** usually gave results slightly better than those with **9** (see Supporting Information).

Next we tried to detect PKA (catalytic subunit) activity. As a solid support, we chose TentaGel and $PEGA_{800}$ resin, since they swell well in aqueous solution and several on-bead enzyme assays using them have already been reported.[3c,17] The substrate peptide synthesized on resin was treated with PKA, base, and dye **4**, followed by observation under a fluorescence microscope. Observation of the beads was performed in glycine/NaOH buffer (pH 9.4), since the fluorescence of **10** was strong and stable in the pH range of 9−10 (see Supporting Information). Our initial experiment with these resins, however, did not work well. Kemptide resin both treated with and without PKA showed little fluorescence (see Supporting Information). Only $PEGA_{1900}$ resin was found to be applicable for PKA enzyme reaction among the resins we tried, probably because PKA was too big to permeate into regular resin, including TentaGel or $PEGA_{800}$. The molecular weight cutoff for $PEGA_{1900}$ is around 70 kDa, whereas the cutoff for $PEGA_{800}$ is around 35 kDa (the molecular weight of PKA is 40 kDa). The fluorescence from the beads treated with PKA increased as the incubation time with PKA was increased (Figure 3a); this result indicated

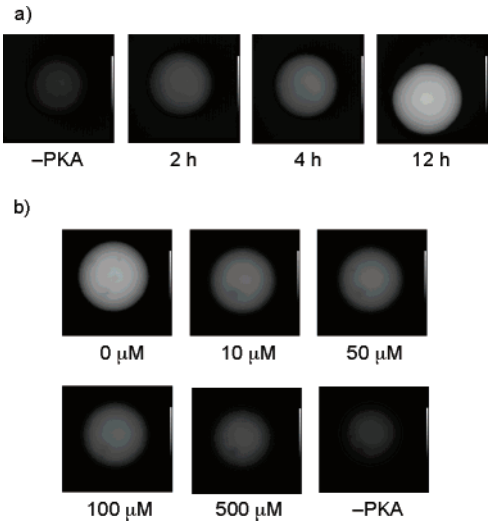

Figure 3. (a) Detection of PKA activity. Beads are incubated with PKA (30 units mL^{-1}) for 2, 4, or 12 h at room temperature and then treated with base and **4**. (b) Effect of IP. Beads were incubated with IP (0−500 μM) and PKA (30 units mL^{-1}) for 10 h at room temperature and then treated with base and **4**. All fluorescence images (ex 360 nm, em >425 nm) were taken in 100 mM glycine/NaOH buffer (pH 9.4).

that the PKA activity could be successfully monitored as fluorescence intensity. Longer incubation time was needed

compared with the solution-phase reaction; the phosphorylation rate of Kemptide on solid support was supposed to be much slower. Although the enzyme reaction rate is decreased, solid-support-based techniques could provide the potential for the simultaneous screening of thousands of enzyme substrates/inhibitors in a single experiment.[3c,17,18] We additionally examined the effect of kinase inhibitor. The PKA-specific inhibitory peptide (IP; Ac-GRTGRRNAI-NH_2)[19] was added at various concentrations (0−500 μM) and incubated with PKA. After the usual procedure, the fluorescence intensity of the beads was observed; it became weaker after addition of the inhibitor, and the fluorescence intensity was inversely correlated with the inhibitor concentration (Figure 3b). This result confirmed that our method can be used for inhibitor screening. Also, these data in Figure 3 can be easily quantified (see Supporting Information).

To test the generality of our method, we next applied it to detect casein kinase I (CK I) activity. CK I substrate peptide (Ac-IGDDDDAYSDTETTEA-resin),[20] which is highly anionic (Kemptide is cationic), was synthesized on $PEGA_{1900}$ beads, and the beads were incubated with CK I or PKA. As a negative control, beads incubated without kinase were also prepared. The subsequent procedure was the same as for the PKA assay, except for the β-elimination conditions; a slightly higher concentration of NaOH was required to complete the reaction. Greater fluorescence intensity was observed on the beads incubated with CK I compared with the negative control and the beads incubated with PKA (Figure 4). Thus, two different kinase activities were selectively detected by our method, suggesting that the method is applicable to various protein kinases.

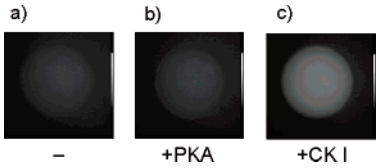

Figure 4. Detection of CK I activity. Beads were incubated (a) without kinase, (b) with PKA (100 units mL^{-1}), or (c) with CK I (100 kunits mL^{-1}) at 30 °C for 20 h. Subsequent treatment was the same as described in the legend to Figure 3.

In summary, we have developed a versatile fluorescence-based assay for serine/threonine kinase. PKA and CK I activities were readily and selectively detected by means of this method. Also, the assay was proven to be suitable for kinase inhibitor screening. Our method is expected to be useful in the high-throughput format, e.g., with microarrays and microplates, and should be a valuable tool for kinase research.

Acknowledgment. This research was supported in part by a Grant-in-Aid for Young Scientists (B) (15790072 and 17790093) to N.U., a Grant-in-Aid for University and Society Collaboration (12793009) to T.H. from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and a Grant-in-Aid for Research in Nagoya City University to N.U. The authors thank Cypress International Co., Ltd. for the donation of $PEGA_{1900}$ resin. We also thank Prof. Naohide Hirashima (Nagoya City University) and Prof. Yasuteru Urano (the University of Tokyo) for helpful comments.

Supporting Information Available: Experimental procedures, spectral data for synthesized compounds, and other detailed results. This material is available free of charge via the Internet at http://pubs.acs.org.

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