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Fluorous-assisted metal chelate affinity extraction technique for analysis of protein kinase activity

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ABSTRACT

We have developed a fluorous affinity-based extraction method for measurement of protein kinase activity. In this method, a fluorescent peptide substrate was phosphorylated by a protein kinase, and the obtained phosphopeptide was selectively captured with Fe(III)-immobilized perfluoroalkyliminodiacetic acid reagent via a metal chelate affinity technique. Next, the captured phosphopeptide was selectively extracted into a fluorous solvent mixture, tetradecafluorohexane and 1*H*,1*H*,2*H*,2*H*-tridecafluoro-1-*n*-octanol (3:1, v/v), using the specificity of fluorous affinity (fluorophilicity). In contrast, the remained substrate peptide in the aqueous (non-fluorous) phase was easily measured fluorimetrically. Finally, the enzyme activity could be assayed by measuring the decrease in fluorescence. The feasibility of this method was demonstrated by applying the method for measurement of the activity of cAMP-dependent protein kinase (PKA) using its substrate peptide (kemptide) pre-labeled with carboxytetramethylrhodamine (TAMRA).

1. Introduction

Functional post-translational modifications (PTMs) of proteins play critical roles in biological systems [1,2]. Among these PTMs, protein phosphorylation is the most important; approximately 30% of proteins in mammalian cells can be phosphorylated by protein kinases, and the phosphorylation is closely related to certain health and/or disease states [3–5]. Therefore, the characterization and identification of protein kinases and measurement of their activity are helpful for understanding the mechanisms and regulatory processes of related diseases in depth and for diagnosis and drug discovery. Various methods have been developed for analysis of protein kinase activity using radioactive, electrochemical, and fluorescence-based detection techniques [6–13] with several affinity methods, including antigen-antibody interactions [6], biotin-avidin affinity [10], and metal chelate affinity [7,11,12]. Although these methods are suitable for a wide variety of kinase assays due to their specificity, the procedures tend to be time consuming and expensive. Therefore, development of simpler and more cost-effective methods that maintain high specificity is needed to improve our ability to assay kinase activity.

As an alternative to the conventional approaches, the affinity between perfluoroalkyl-containing compounds (*i.e.*, fluorous compounds), or fluorophilicity, has recently been applied in novel fractionation methods for analysis of biological samples [14–16]. This fluorous affinity method can be used for highly selective enrichment and purification of perfluoroalkylated (fluorous) compounds to the exclusion of non-fluorous compounds via their analog solvents or materials, *e.g.*, fluorous biphasic liquid-liquid extraction and/or fluorous stationary solid-phase separation systems [17,18]. We previously reported the development of a newly selective liquid chromatographic (LC) analysis method for biogenic-related compounds using the fluorous technology [19–23]. These methods were based on the derivatization of analytes with perfluoroalkylated reagents and the selective retention and separation of the derivatives by a perfluoroalkyl-modified stationary phase LC column. Additionally, we also recently developed a novel fluorous biphasic liquid-liquid extraction method for nucleotides utilizing a non-covalent interaction, such as ion-pairs, with a fluorous reagent [24]. Thus, ion pairs between phosphate groups on nucleotides and the amine-containing fluorous reagent were successfully formed and could be selectively extracted using a fluorous solvent, such as a mixture of tetradecafluorohexane (TDFH) and 1*H*,1*H*,2*H*,2*H*-tridecafluoro-1-*n*-octanol (TFO), without undergoing a chemical derivatization reaction.

In the present study, we developed a selective assay method for protein kinase activity utilizing a fluorous biphasic system in

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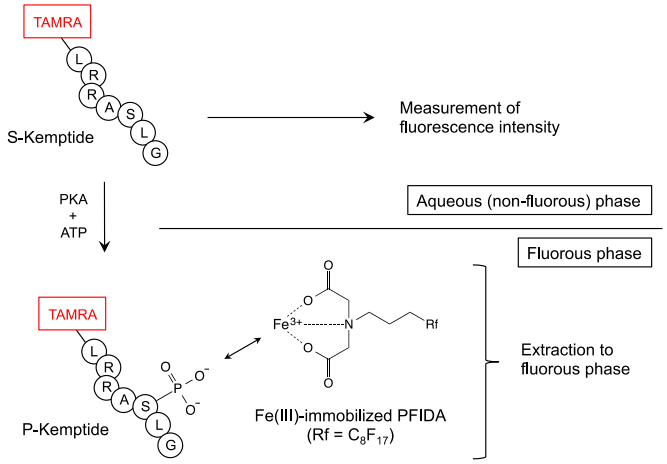

Fig. 1. Concept of the PKA activity assay using the fluorous-metal chelate affinity extraction method.

conjunction with a metal chelate affinity technique. Metal chelate affinity has been applied as a selective enrichment technique for phosphate-containing compounds, primarily phosphorylated peptides [25,26]. In this technique, iminodiacetic acid (a tridentate metal chelator) and nitrilotriacetic acid (a quadradentate metal chelator) were used as chelating ligands, and their amino groups and carboxylic groups immobilized metal ions, such as Fe(III), Ni (II), Zr(IV), or Ca(III), by chelation. The coordination sites of immobilized metal ions could then capture negatively charged phosphate groups. In this study, we synthesized an iminodiacetic acid-containing perfluoroalkyl reagent, *e.g.*, perfluoroalkyliminodiacetic acid (PFIDA), whose metal chelating property has been reported previously [27], and Fe(III) was then immobilized to the synthesized PFIDA. Because the Fe(III)-immobilized PFIDA could not only recognize the phosphate groups but also exhibit specific fluorous affinity, this potential allowed us to use this assay for analysis of protein kinase activity by selectively extracting enzymatically phosphorylated peptides. Our strategy using Fe(III)-immobilized PFIDA is shown in Fig. 1. In this method, cAMP-dependent protein kinase (PKA) and kemptide (LRRASLG), whose *N*-terminal was pre-labeled with carboxytetramethylrhodamine (TAMRA), were used as a model enzyme and substrate peptide, respectively. After enzymatic reaction, the phosphorylated kemptide (P-kemptide) could be selectively excluded with Fe(III)-immobilized PFIDA from the reaction mixture (aqueous) to fluorous solvent by fluorous biphasic liquid-liquid extraction. Because the substrate, *i.e.*, the non-phosphorylated kemptide (S-kemptide) labeled with TAMRA, remained in the reaction mixture, the PKA activity could be assayed by comparison of the fluorescence intensity of the mixture in the non-fluorous phase before and after the enzymatic reaction. For measurement of the fluorescence intensity of (S-kemptide), we used a Nanodrop 3300 fluorescence spectrometer (ThermoFisher Scientific, San Jose, CA, USA). The Nanodrop 3300 allowed us to use samples of less than 2-μL in volume, thereby enabling a significant reduction in the total sample volume. Because of its simplicity and rapidity, this device has been used for protein concentration and enzyme activity assays as an alternative to conventional spectrofluorometric methods [28,29]. We carried out the measurement of the samples obtained after enzymatic reaction with different concentrations of PKA and then demonstrated that our novel method employing a simple fluorous biphasic system and Nanodrop fluorometry measurement could be used for analysis of PKA activity without time-consuming steps.

2. Experimental

2.1. Reagents and materials

TAMRA-labeled kemptide (S-kemptide), LRRASLG) and its phosphorylated product (P-kemptide), LRRApSLG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Adenosine triphosphate (ATP), PKA, and PKA buffer were obtained from New England Biolabs Inc. (Beverly, MA, USA). Trifluoroacetic acid (TFA) was purchased from Kanto Chemical (Tokyo, Japan). 4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-Heptadecafluoroundecylamine (HFUA), TDFH, and TFO were purchased from Fluorous Technologies (Pittsburgh, PA, USA), Wako Pure Chemical (Osaka, Japan), and Tokyo Chemical Industry (Tokyo, Japan), respectively. PFIDA was synthesized from HFUA (Supplementary material). Deionized water was purified using a Millipore EQG system (Billerica, MA, USA) and was used to prepare all aqueous solutions. All other organic solvents from Wako Pure Chemical were of LC-grade and used as received. Because organic solvents are toxic if exposed to the eyes, lungs, or skin, they should be carefully handled in accordance with the latest material safety data sheets.

2.2. Preparation of Fe(III)-immobilized PFIDA

PFIDA solution was prepared by mixing TDFH and TFO (3:1, v/v) as a fluorous solvent, and a saturated $FeCl_3$ solution in 50% (v/v) aqueous methanol was added. The mixture was then shaken at room temperature for 30 min After centrifugation at 17,500*g* for 10 min, the supernatant (non-fluorous phase) was discarded. Dyed sediment solution (fluorous phase) by chelating Fe(III) to PFIDA was washed with 50% (v/v) aqueous methanol until the supernatant became colorless. Then, after the fluorous solvent was evaporated under reduced pressure to dryness, the obtained Fe (III)-immobilized PFIDA was dissolved again in a mixture of TDFH and TFO (3:1, v/v) to the required concentrations before use.

2.3. Measurement of PKA activity with fluorous biphasic extraction

Seventy-five microliters of a mixture of 40 μM (S-kemptide) and PKA was prepared using 1 × PKA buffer [50 mM Tris–HCl buffer (pH 7.5) with 10 mM $MgCl_2$] and placed in a 0.5-mL screw cap tube. Next, 75 μL of 400 μM ATP in 1 × PKA buffer was added, and the solution was incubated at 30 °C for 2 h in order to transform (S-kemptide) to (P-kemptide). After incubation, 100 μL of 0.2% TFA in acetonitrile was added. To an aliquot of this enzymatic solution (50 μL), 20 μL of 40 mM Fe(III)-immobilized PFIDA in fluorous solvent, TDFH and TFO (3:1, v/v), was added, and the mixture was shaken at room temperature for 10 min After centrifugation at 17,500 × *g* for 10 min for demulsification, the fluorescence intensity of the supernatant, representing the unreacted substrate (S-kemptide)) in the aqueous phase, was measured with a Nanodrop 3300 fluorescence spectrometer. The samples (2 μL) were illuminated with white LED excitation, and the fluorescence emission data was obtained in triplicate measurements on the same sample by scanning from 550 to 620 nm.

3. Results and discussion

In general, the fluorous biphasic liquid-liquid extraction method has been used to extract heavily fluorous compounds (which contain several fluoroalkyl chains with 39 or more fluorines), whereas lightly fluorous compounds (which contain moderate fluoroalkyl chains with 9–17 fluorines) cannot be extracted because of their low solubility in representative fluorous solvents, such as perfluoroalkanes (*e.g.*, TDFH, perfluoromethylcyclohexane,