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In-gel protein phosphatase assay using fluorogenic substrates

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ABSTRACT

We developed a method for the detection of phosphatase activity using fluorogenic substrates after polyacrylamide gel electrophoresis. When phosphatases such as Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP), protein phosphatase 2C (PP2C), protein phosphatase 5 (PP5), and alkaline phosphatase were resolved by polyacrylamide gel electrophoresis in the absence of SDS and the gel was incubated with a fluorogenic substrate such as 4-methylumbelliferyl phosphate (MUP), all of these phosphatase activities could be detected *in situ*. Although 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) as well as MUP could be used as a fluorogenic substrate for an in-gel assay, MUP exhibited lower background fluorescence. Using this procedure, several fluorescent bands that correspond to endogenous phosphatases were observed after electrophoresis of various crude samples. The in-gel phosphatase assay could also be used to detect protein phosphatases resolved by SDS–polyacrylamide gel electrophoresis. In this case, however, the denaturation/renaturation process of resolved proteins was necessary for the detection of phosphatase activity. This procedure could be used for detection of renaturable protein phosphatases such as CaMKP and some other phosphatases expressed in cell extracts. The present fluorescent in-gel phosphatase assay is very useful, since no radioactive compounds or no special apparatus are required.

A variety of biological processes in animals, plants, and microorganisms are regulated by protein phosphorylation [1]. Intracellular signaling networks are known to be constructed on the basis of the subtle balance between phosphorylation by protein kinases and dephosphorylation by 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.

Previously, we developed an in-gel protein kinase assay after separation of cellular proteins in SDS–PAGE[1] [2,3]. After that, we also developed an in-gel protein phosphatase assay [4], and we discovered Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP) from rat brain using this technique [5]. These in-gel assays are very useful for the detection of novel protein kinases and phosphatases expressed in tissue and cell extracts, but radioactive materials such as [γ-^{32}P]ATP are required for the assays. Therefore, to carry out these experiments, we always had to consider the short half-life of [γ-^{32}P]ATP and the need for a special facility for radioactive materials. In the previous studies on protein phosphatases, not only phosphorylated proteins but also chromogenic compounds such as *p*-nitrophenyl phosphatase were used as substrates [6,7]. In other cases, alkaline phosphatase, protein phosphatase 1 (PP1) [8], and protein phosphatase 5 (PP5) [9] were assayed using fluorogenic substrates such as 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) and 4-methylumbelliferyl phosphate (MUP). The sensitivity of assays using fluorogenic substrates is often superior to that of chromogenic substrates. Therefore, we examined whether or not these fluorogenic substrates could be used for detection of various protein phosphatases after polyacrylamide gel electrophoresis.

In the present study, we demonstrated that both MUP and DiFMUP could detect various protein phosphatases in tissue extracts after separation by polyacrylamide gel electrophoresis in the absence of SDS. Furthermore, we showed that protein phosphatases such as CaMKP could be detected in SDS–polyacrylamide gels when these enzymes were properly renatured *in situ*.

Materials and methods

Materials

Alkaline phosphatase from calf intestine (CIP) was obtained from Roche Diagnostics. DiFMUP and MUP were purchased from Invitrogen and Sigma, respectively. These fluorogenic substrates were dissolved in dimethyl sulfoxide in a concentration of

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[1] *Abbreviations used:* CaMKP, Ca^{2+}/calmodulin-dependent protein kinase phosphatase; CIP, alkaline phosphatase from calf intestine; DiFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; MUP, 4-methylumbelliferyl phosphate; Native-PAGE, native polyacrylamide gel electrophoresis; PP1, protein phosphatase 1; PP5 protein phosphatase 5; PP2C, protein phosphatase 2C; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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10 mM and stored at −30 °C until use. Polyclonal antibody against rat CaMKP was prepared using synthetic peptide corresponding to a carboxyl-terminal sequence as described previously [10].

Recombinant phosphatases

Recombinant rat CaMKP and PP2Cα were expressed in *Escherichia coli* and purified as described previously [11]. Rat PP5 was cloned and expressed in *E. coli* as follows. The cDNA of rat PP5 (Accession No. X77237) was amplified using sense (5′-GAA TTC ATG GCG ATG GCG GAG GGC GA-3′) and antisense (5′-CTC GAG CAT CAT TCC TAG CTG CAG CAG CG-3′) primers and rat brain 3′-RACE ready cDNA library as a template. The amplified fragment was subcloned into EcoRI–XhoI sites of pET-23a(+) (Novagen) and the recombinant plasmid was designated as pETrPP5. *Escherichia coli* BL21(DE3) cells transformed with pETrPP5 were grown at 37 °C for 16 h in 1 ml of medium A (LB medium containing 100 μg/ml ampicillin) with shaking. The culture was then transferred to a 300-ml flask containing 100 ml of medium A and incubated with shaking at 25 °C to an A_{600} of 1.0, and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. After 12 h at 25 °C, the bacteria were harvested by centrifugation and suspended in 10 ml of buffer A (20 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 0.05% (v/v) Tween 40, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). After sonication, cell debris was removed by centrifugation (20,000*g*) for 10 min, and the supernatant was loaded onto a HiTrap Chelating HP column (1 ml; GE Healthcare) pre-equilibrated with buffer A. The column was subsequently washed with 10 ml of buffer A, 10 ml of buffer A containing 20 mM imidazole, and 10 ml of buffer A containing 50 mM imidazole. Following this, the column was eluted with buffer A containing 200 mM imidazole. The purified fractions were pooled and used for phosphatase assay.

Rat tissue extract

Tissue extracts were prepared from male Wistar rats (Japan SLC). Various tissues from rat were suspended in 5 vol of homogenizing buffer containing 5 mM Tris–HCl (pH 7.5), 0.5 mM EGTA, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, and homogenized by a Teflon/glass homogenizer or by Polytron (Kinematica AG). The homogenates were centrifuged at 20,000*g* at 2 °C for 30 min, and the supernatants obtained were used as crude extracts. Protein concentration was determined by the method of Bensadoun and Weinstein using bovine serum albumin as a standard [12].

SDS–PAGE, Native-PAGE, and Western blotting

SDS–PAGE was carried out essentially according to the method of Laemmli [13] in slab gels consisting of a 10% (w/v) acrylamide separating gel and a 3% (w/v) stacking gel. Native-PAGE was performed using essentially the same procedure as SDS–PAGE except that SDS was omitted from the sample buffer, electrode buffer, and polyacrylamide gels. SDS–PAGE was carried out at room temperature, but Native-PAGE was done at 4 °C to prevent loss of phosphatase activity. Western blotting was performed essentially as described previously [14].

In-gel protein phosphatase assay

In the case of Native-PAGE, the electrophoresed gel was directly soaked in 3 ml of reaction mixture containing 50 mM Tris–HCl (pH 8.0), 0.1 mM EGTA, 0.01% (v/v) Tween 20, 2 mM dithiothreitol, 20 mM $MnCl_2$, and 0.5 mM MUP (or DiFMUP). The gel was incubated at 37 °C for 15 min, and fluorescent bands were observed by a transilluminator with an excitation wavelength at 365 nm. Incubations longer than 30 min are not recommended, since it will cause diffusion of fluorescent products formed in gels.

In the case of SDS–PAGE, the denaturation/renaturation process is necessary before detection of phosphatase activity by fluorogenic substrates. After electrophoresis, SDS was removed by washing the gel with two changes of 100 ml each of 20% (v/v) 2-propanol in 50 mM Tris–HCl (pH 7.5) for 1 h at room temperature. Then the gel was treated with two changes of 50 ml of a denaturation buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, and 8 M urea (or 6 M guanidine HCl) for 1 h at room temperature, to denature resolved proteins in gel. Then the gel was put into 100 ml of a renaturation buffer containing 50 mM Tris–HCl (pH 7.5), 0.02% (v/v) Tween 20, 20 mM 2-mercaptoethanol, and 1 mM $MgCl_2$ (or $MnCl_2$) and gently shaken at 4 °C. This renaturation process was continued for 16–20 h with 5 changes of renaturation buffer. After the treatment, the gel was incubated with the reaction mixture containing fluorogenic substrates as above.

Results

MUP and DiFMUP as fluorogenic substrates for phosphatases

In the previous study, we developed an in-gel protein phosphatase assay using ^{32}P-labeled peptides [4], and we discovered a novel protein phosphatase, CaMKP, in rat brain using this technique [5]. In this study, therefore, we attempted to develop a simple in-gel protein phosphatase assay without using radioactive materials. Fluorogenic substrates such as MUP and DiFMUP have been used as substrates for phosphatases such as alkaline phosphatase, PP1 [8], and PP5 [9] in solution-based assays. Both MUP and DiFMUP are known to be converted to fluorescent products when they are hydrolyzed. When examined, recombinant CaMKP and PP2C obtained in *E. coli* expression system also hydrolyzed both MUP and DiFMUP, and produced fluorescent products (data not shown).

In-gel phosphatase assay after Native-PAGE

In-gel phosphatase assay was first examined after polyacrylamide gel electrophoresis in the absence of SDS, namely Native-PAGE. Varying amounts of CIP were resolved on Native-PAGE at 4 °C, and then the gels were incubated in the reaction buffers each containing fluorogenic substrates. CIP showed a single clear fluorescent band and fluorescence intensity gradually increased in parallel with the amount of phosphatase loaded on the gels (Fig. 1). DiFMUP exhibited higher sensitivity than MUP when CIP was detected in gel (Fig. 1). When protein phosphatases such as CaMKP, PP2C, and PP5 were analyzed in gel, these phosphatases exhibited different reactivities against these substrates (Fig. 2A).

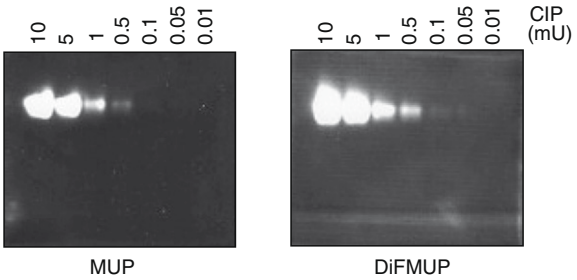

Fig. 1. In-gel assay of alkaline phosphatase. Indicated amounts of CIP were resolved on the Native-PAGE. After electrophoresis at 4 °C, the gel was incubated with the reaction mixture containing either MUP (left panel) or DiFMUP (right panel). For comparison of sensitivity, all experiments were carried out under the same conditions except that different fluorogenic substrates were used.