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In-gel protein phosphatase assay using fluorogenic substrates

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Short title: Fluorescent in-gel phosphatase assay

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ABSTRACT

We developed a method for 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 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, denaturation/renaturation process of resolved proteins was necessary for detection of phosphatase activity. This procedure could be used for detection of renaturable protein phosphases 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.

Keywords: fluorogenic substrate; in-gel assay; Native-PAGE; protein phosphatase; SDS-PAGE.

Introduction

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 regulatory mechanisms of signal transduction by protein phosphorylation, it is important to develop techniques to detect and analyze both protein kinases and protein phosphatases.

Previously, we developed in-gel protein kinase assay after separation of cellular proteins in SDS-PAGE [2, 3]. After that, we also developed 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 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 about short half life of [γ-^{32}P]ATP and special facility for radioactive materials. In the previous studies on protein phosphatases, not only phosphorylated proteins but also choromogenic compounds such as *p*-nitrophenyl phosphatate have been used as substrates [6, 7]. In other cases, alkaline phosphatase, protein phosphatase 1 (PP1) [8], and protein phosphatase 5 (PP5) [9] have been 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 10 mM and stored at -30°C until use. Polyclonal antibody against rat CaMKP was prepared using synthetic peptide corresponding to carboxyl-terminal sequence as described previously [10].

Recombinant phosphatases

Reconbinant rat CaMKP and PP2Cα were expressed in *Eschelichia 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 *Eco*RI-*Xho*I

sites of pET-23a(+) (Novagen) and the recombinant plasmid was designated as pETrPP5. *E. 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 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 volumes 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 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 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 transilluminator with excitation wavelength at 365 nm. Longer incubation than 30 min is not recommended, since it will cause diffusion of fluorescent products formed in gels.

In case of SDS-PAGE, 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 substates as above.

Results

MUP and DiFMUP as fluorogenic substrates for phosphatases

In the previous study, we developed in-gel protein phosphatase assay using ^{32}P-labeled peptides [4], and we discovered novel protein phosphatase, CaMKP, in rat brain using this technique [5]. In this study, therefore, we attempted to develop 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 assay. 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 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). CaMKP and PP2C showed clear fluorescent bands with MUP, while PP5 and alkaline phosphatase showed more significant fluorescent bands when DiFMUP was used (Fig. 2A). These results suggest that MUP appears to be better fluorogenic substrate for detection of a wide variety of protein phosphatases. When rat tissue extracts were analyzed by in-gel protein phosphatase assay using MUP, various phosphatases with different mobilities on Native-PAGE could be detected (Fig. 2B).

In-gel phosphatase assay after SDS-PAGE

In our previous study, we detected CaMKP activity in polyacrylamide gels after separation on SDS-PAGE [4]. Here, we examined whether CaMKP could be detected by fluorescent in-gel assay after separation by SDS-PAGE. When CaMKP was resolved on SDS-PAGE and reacted with MUP or DiFMUP directly, no fluorescent band could be observed even as much as 5 μg of CaMKP was applied (data not shown). Next, we examined denaturation/renaturation treatment before detecting phosphatase activity in gel. The gel was treated with 6 M guanidine HCl or 8 M urea at room temperature for 1 h, and then incubated in the renaturation buffer at 4°C for 16-20 h. After renaturation process, the gel was soaked in the reaction buffer containing either MUP or DiFMUP.

Although both gels showed single fluorescent band corresponding to CaMKP, MUP showed clearer fluorescent band in a dark background than DiFMUP (Fig. 3). DiFMUP exhibited somewhat brighter background fluorescence throughout the gel as compared to MUP, presumably due to trace contamination of degradation products in the reagent. Therefore, we mainly used MUP as a substrate for in-gel phosphatase assays after SDS-PAGE.

Optimum conditions for in-gel detection of CaMKP were investigated. Denaturation treatment either by 8M urea or 6 M guanidine HCl showed essentially similar results (not shown), as in case of previous in-gel phosphatase assay [4]. Concerning metal ions, Mn^{2+} ion, but not Mg^{2+}ion, included in the reaction mixture was essential for detection of phosphatase activity in gels (Fig. 4B). In contrast, Mg^{2+} ion showed somewhat more efficient effect on the renaturation of CaMKP than Mn^{2+} ion did (Fig. 4A). Under the conditions, 50 ng of CaMKP could be detected.

This procedure could be used for detection of renaturable protein phosphatases after SDS-PAGE. When recombinant CaMKP was expressed in *E. coli*, it was recovered not only in a soluble fraction but also in an insoluble fraction as in inclusion body. Both CaMKP obtained in soluble fraction and insoluble fraction could be equally detected in gel with essentially the same reactivity (Fig. 5A). When rat tissue extracts were analyzed with this procedure, restricted number of phosphatases could be detected. Fluorescent protein bands corresponding to 52-kDa and 40-kDa phosphatases were detected in brain, while 40-kDa phosphatase was detected in all the tissues examined (Fig. 5B).

Discussion

In-gel assay techniques are widely used for detection of various functional enzymes following electrophoretic separation in gels [15]. In our previous study, we developed in-gel protein phosphatase assay using ^{32}P-labeled peptides as substrates included in SDS-polyacrylamide gels [4]. Using this technique, we discovered novel protein phosphatase in rat brain and designated as CaMKP [5]. Although the in-gel phosphatase assay was very useful technique to detect protein phosphatases in crude extracts, it was inconvenient because we had to label proteins or peptides with [γ-^{32}P]ATP to obtain radioactive substrates. Therefore, we have been attempting to develop in-gel phosphatase assay without using radioactive materials. Recently, MUP and DiFMUP were reported to be used for the fluorometoric solution-based assay of PP5 [9]. In the present study, therefore, we attempted to introduce these fluorogenic substrates for detection of protein phosphatases in polyacrylamide gels.

Chromogenic substrate such as *p*-nitrophenyl phosphate has often been used for the assay of protein phosphatases in solution [6, 7]. However, we found that this substrate was not applicable to in-gel phosphatase assay (data not shown). There may be two possible explanations why *p*-nitrophenyl phosphate is not suitable substrate for in-gel phosphatase assay. In our experiments using solution-based assay, 10-times less amount of PP2C could be readily be detected by fluorescent assay using MUP as compared to the phosphatase assay using *p*-nitrophenyl phosphate. These results indicate that MUP assay shows more than 10-times higher sensitivity than *p*-nitrophenyl phosphate assay. Another advantage of MUP and DiFMUP for in-gel assay is their lower solubility in aqueous solution. Fluorescent products of MUP and DiFMUP tend to stay *in situ* because of their insolubility in water, which minimize diffusion of

fluorescent compounds formed in gel. In contrast, *p*-nitrophenol, a product of *p*-nitrophenyl phosphate, may easily be diffused away from the gel even if it was formed in gels. In case of aforementioned fluorogenic substrates, however, it should be noted that longer incubation will cause slow diffusion of fluorescent products from the gels.

We found that protein phosphatases such as CaMKP, PP2C, and PP5 could be detected directly after Native-PAGE just by incubating with these fluorogenic substrates. Alkaline phosphatases and PP5 reacted more efficiently with DiFMUP than MUP, which was consistent with the previous experiments performed in solution [8, 9]. DiFMUP has been reported to be superior to MUP, especially, in the assay of phosphatases under neutral or acidic pH conditions. The differences in fluorescence intensity may be attributed to the differences in p*K*a values between DiFMUP and MUP. However, we found that CaMKP and PP2C reacted more efficiently with MUP than DiFMUP in the in-gel assay at pH 8. These results may reflect the differences in the substrate specificities of the phosphatases used for the assay. Under the present conditions, DiFMUP exhibited higher background fluorescence throughout the gel, presumably due to trace amount of contamination of fluorescent products in commercially available DiFMUP. In addition, DiFMUP is much more expensive reagent (approximately 150 times more expensive) than MUP. These results, taken together, suggest that MUP is more suitable substrate for the in-gel assay for detection of a wide variety of protein phosphatases in crude cell extracts.

In-gel phosphatase assay using fluorogenic substrates could be applied for detection of phosphatases after SDS-PAGE. With this procedure, CaMKP and some other phosphatases that could be renatured in gel were detected. In our previous study, we

detected protein phosphatases with molecular masses of 74 kDa, 58 kDa, and 52 kDa in rat brain extract when ^{32}P-labeled phosphopeptide was used as a substrate [4]. In the present study, we could detect phosphtases of 52 kDa and 40 kDa in rat brain (Fig. 5B). These results indicate that different patterns of phosphatase activity observed in the present and previous studies may reflect the differences in the substrate specificities of different phosphatases. Therefore, development of the fluorescent in-gel assay does not mean to take place of the previous in-gel assay, because different phosphatases could be detected by the different substrates used for the assay.

The present paper reports the fluorescent in-gel phosphatase assay either after SDS-PAGE or Native-PAGE. In-gel assay after SDS-PAGE is useful for detection of restricted number of renaturable phosphatases including CaMKP in crude extracts. On the other hand, in-gel phosphatase assay after Native-PAGE may be used for the analysis of the changes in the activities of various phosphatases expressed in the cells under varying situations.

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Footnotes (for the title page)

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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.

Figure legends

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 the different fluorogenic substrates were used.

Fig. 2. In-gel detection of phosphatases after separation by Native-PAGE. (A) CaMKP (5 μg), PP2C (2.5 μg), PP5 (2.5 μg), and CIP (1 mU) were resolved on Native-PAGE and phosphatase activities detected by MUP (left panel) or DiFMUP (right panel). (B) Crude extracts (100 μg) from various rat tissues, CaMKP (5 μg), and PP2C (2.5 μg) were electrophoresed as indicated and phosphatase activities detected by MUP.

Fig. 3. In-gel detection of CaMKP after SDS-PAGE. Recombinant rat CaMKP (1 μg) was electrophoresed on SDS-PAGE. After electrophoresis, gel was cut into three pieces and one of them stained with Coomassie brilliant blue (A). Other gels were treated with 8 M urea and then incubated with renaturation buffer at 4°C for 16 h. After renaturation process, CaMKP activity was detected by 0.5 mM MUP (B) and DiFMUP (C).

Fig. 4. Effect of metal ions on renaturation and phosphorylation of CaMKP in gel. Varying amounts of CaMKP (1000, 500, 200, 100, 50, and 20 ng) were resolved by SDS-PAGE. (A) The gels were treated with 8M urea and then incubated with the renaturation buffer containing 1 mM $MgCl_2$ (middle panel), 1 mM $MnCl_2$ (right panel),

or without metal ion (left panel) for 16 h at 4°C. After renaturation, CaMKP activity was detected by reaction mixture containing 0.5 mM MUP. (B) The gels were treated with 8 M urea and incubated with renaturation buffer containing 1 mM $MgCl_2$. Then the gels were incubated with the reaction mixture containing 0.5 mM MUP and 20 mM $MgCl_2$ (middle panel), 20 mM $MnCl_2$ (right panel), or without metal ion (left panel).

Fig. 5. Detection of protein phosphatases in gel after SDS-PAGE. (A) *E. coli* expressing CaMKP and mock transfected *E. coli* were harvested, homogenized, and centrifuged. Soluble fractions (S) and precipitated fractions (P) were dissolved in SDS sample buffer, and were electrophoresed on SDS-PAGE. After SDS-PAGE, the gels were analyzed by Western blotting with anti-CaMKP antibody (left panel) or by in-gel phosphatase assay using MUP as a substrate (right panel). An arrowhead and asterisk indicate CaMKP and an endogenous phosphatase expressed in *E. coli*, respectively. (B) CaMKP (1 μg), PP2C (1 μg), and crude extracts (100 μg) from rat tissues as indicated were resolved by SDS-PAGE, renatured in gel, and detected by in-gel phosphatase assay using MUP as a substrate.

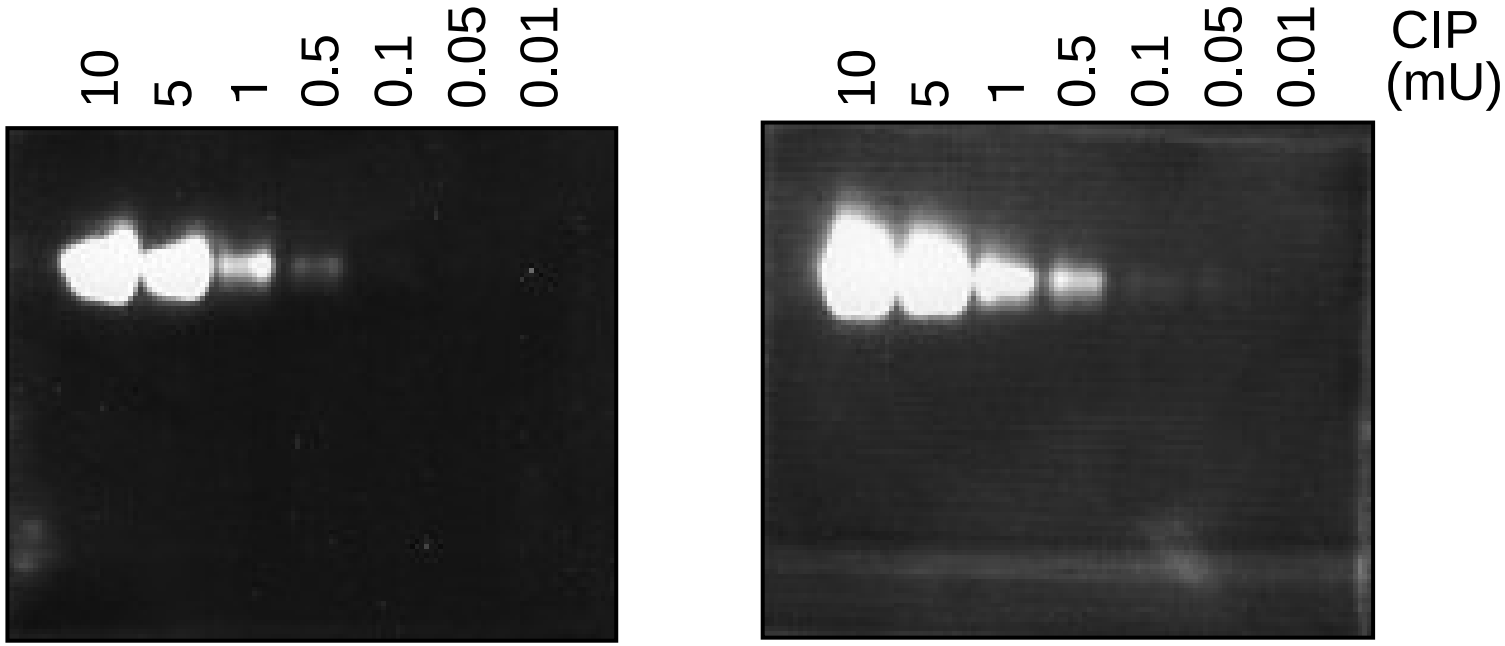


Fig.1

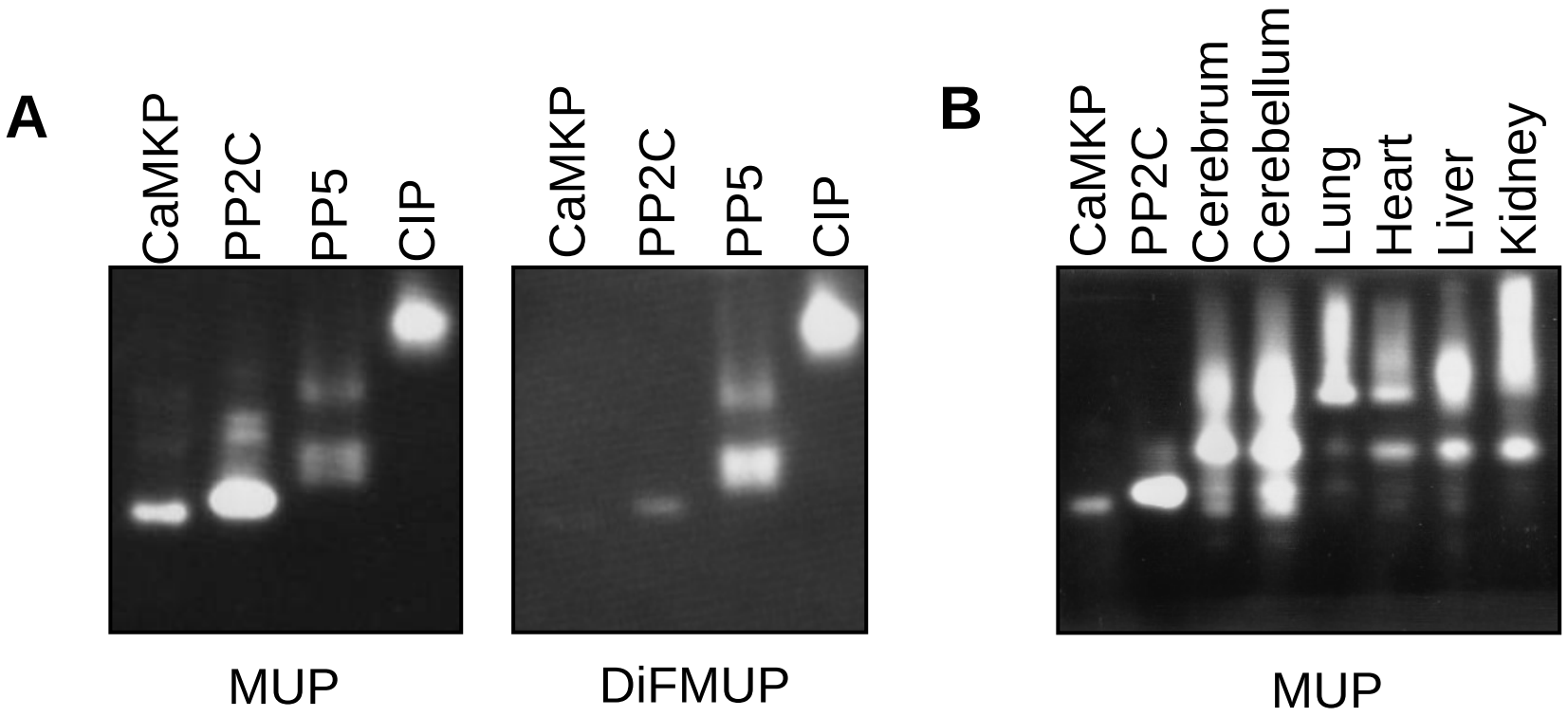
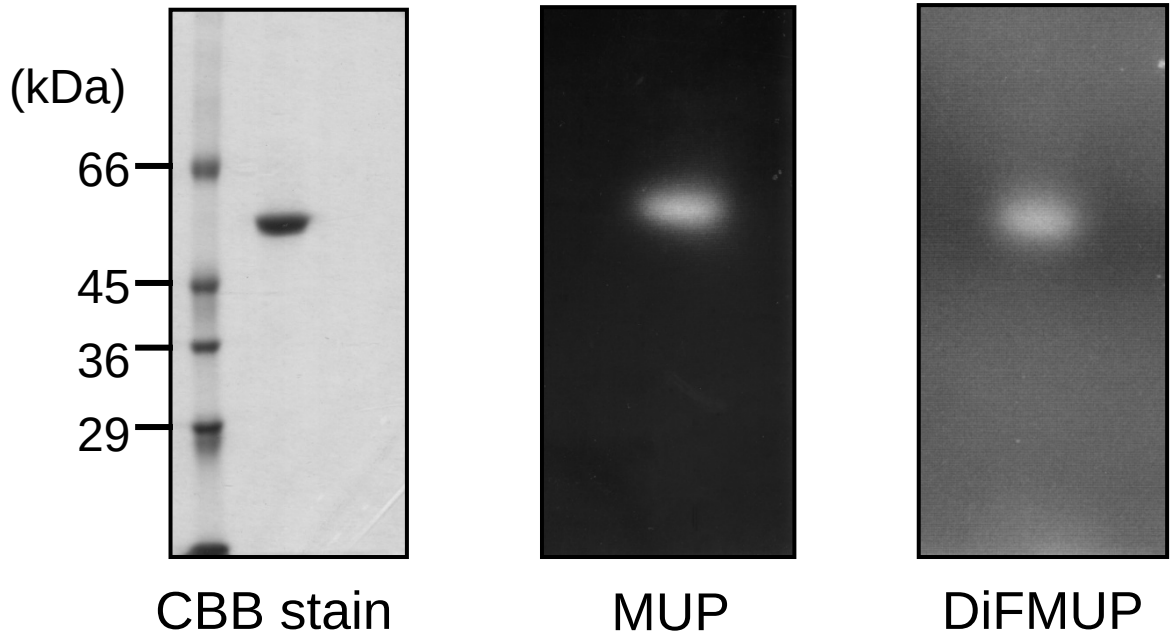


Fig.2



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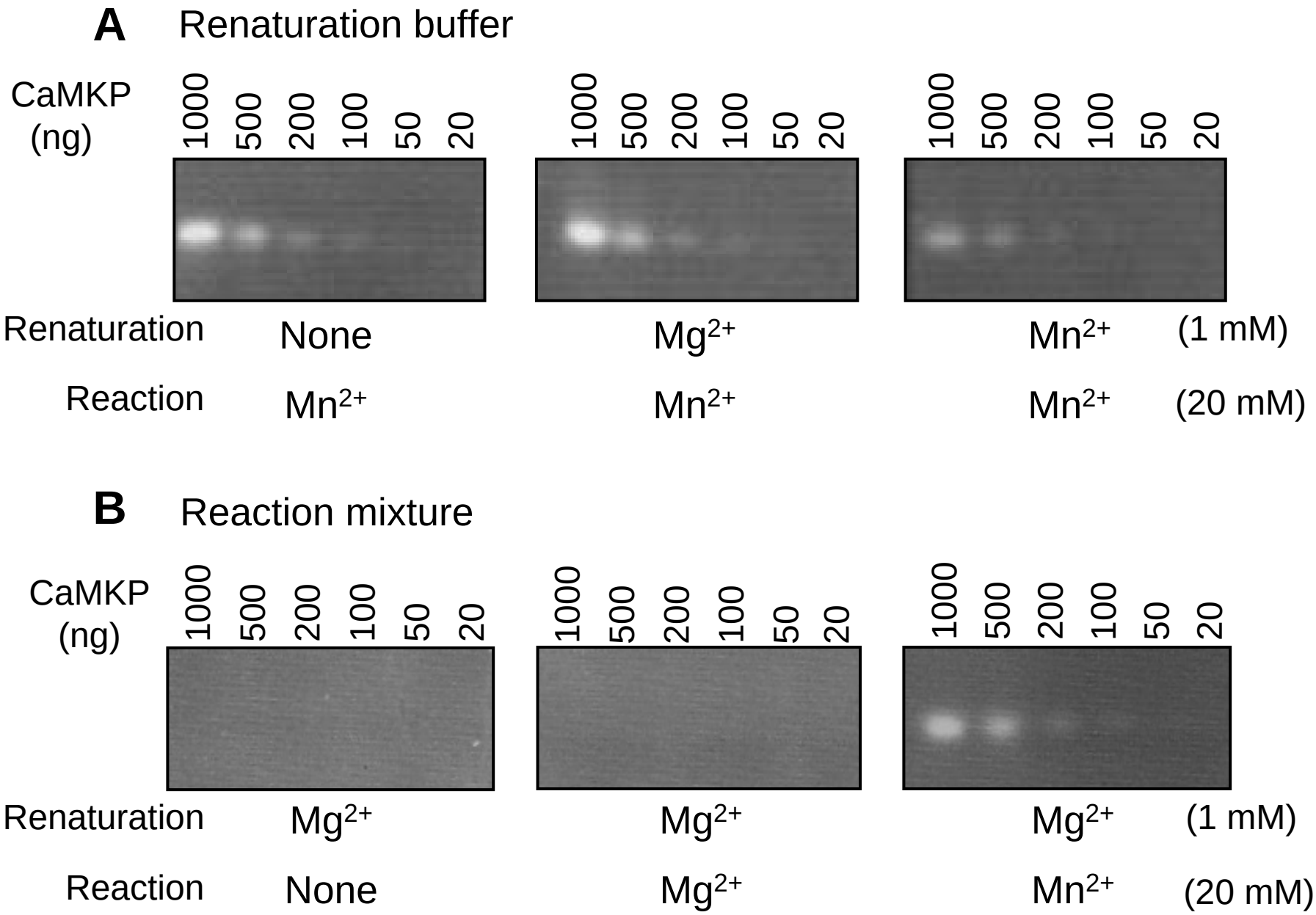

Fig.4

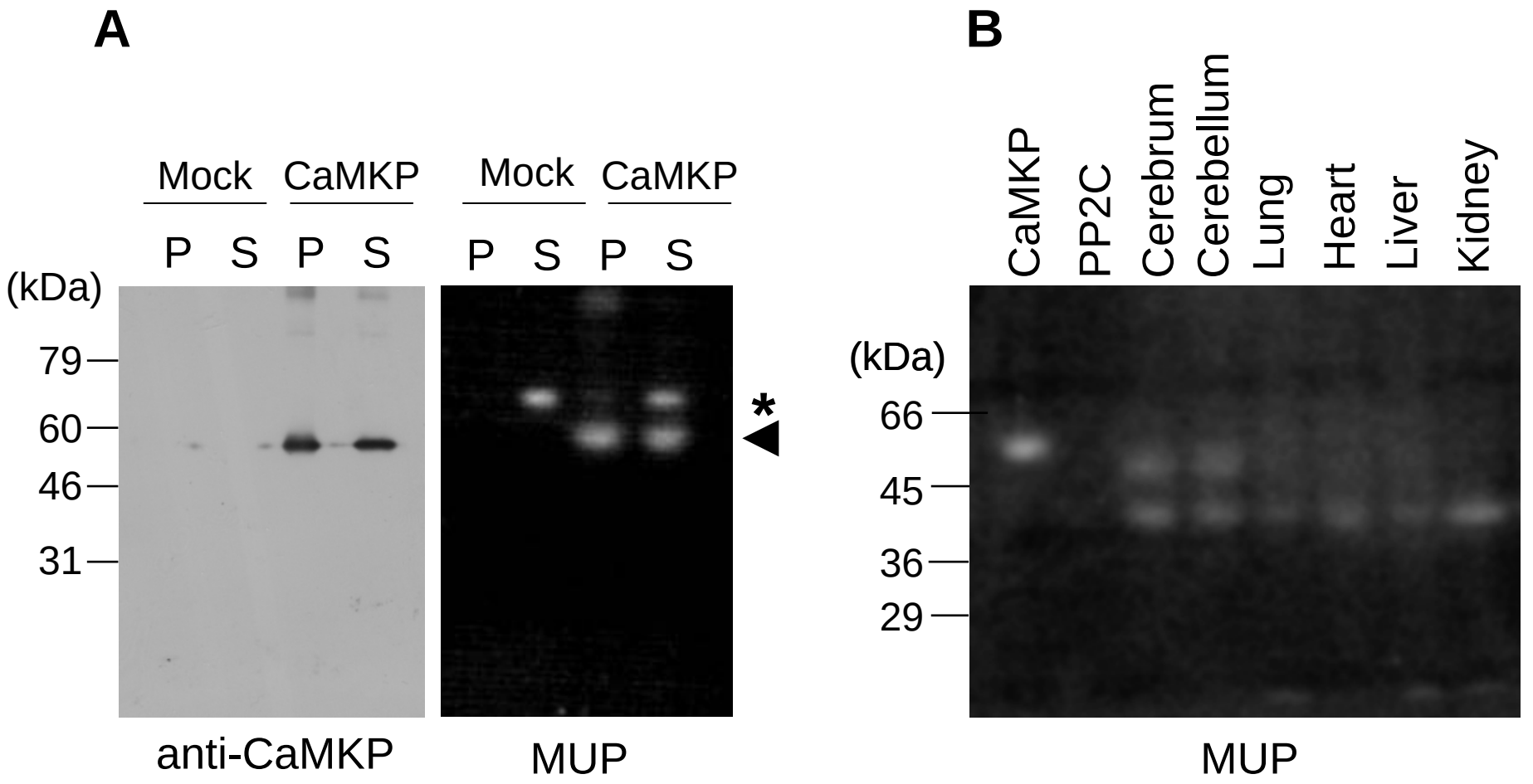

Fig.5