Notes & Tips

Separation of a phosphorylated-His protein using phosphate-affinity polyacrylamide gel electrophoresis

Categories: Electrophoretic Techniques

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Abbreviations used: acrylamide-pendant Phos-tag ligand, *N*-(5-(2-acryloylaminoethylcarbamoyl)pyridin-2-ylmethyl)-*N*,*N*',*N*'-tris(pyridine-2-yl-methyl)1,3-diaminopropan-2-ol; CBB, Coomassie Brilliant Blue; HK, histidine kinase; Mn^{2+}–Phos-tag, dinuclear Mn^{2+}-complex with Phos-tag ligand; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; ThkA, *Thermotoga maritima* HK-TM1359 (pir id: H72262).

It is important to visualize phosphorylated proteins in evaluating diverse signal transductions in biological systems. Kinoshita, *et al*. recently reported the separation of phosphorylated from unphosphorylated proteins using a novel phosphate-affinity SDS-PAGE technique [1]. The affinity gel contained a phosphate-binding tag molecule, Phos-tag, a dinuclear Mn^{2+} complex with an acrylamide-pendant Phos-tag ligand (commercially available at www.phos-tag.com). The principle of this method is as follows; phosphorylated proteins move slower in SDS-PAGE than the corresponding unphosphorylated molecules, since the phospho-group interacts with the Mn^{2+}–Phos-tag ligand in the gel.

This novel technique was successfully used to detect some proteins containing phosphorylated Ser, Thr, and Tyr residues (O-phosphates) [1]. In addition, since the standard free energy of the N-P bond of N-phosphorylated His is large, –42 kJ/mol, the phosphorylated His has the potential to serve as an intermediate in phospho-transfer reactions to other amino acids [2]. Therefore, in addition to Ser/Thr and Tyr kinases, His kinases (HK) play a crucial role as a sensor apparatus in signal transduction, the two-component regulatory system, in prokaryotes, fungi, and plants [3]. In response to intra- and extra-cellular signals (*e.g.,* nutrients, light, and oxygen), HK promotes autophosphorylation at a conserved His residue using ATP, and the phosphoryl group is subsequently transferred to a unique Asp residue in a response regulator protein. The detection of phosphorylated HK is very important, in terms of measuring the signal response by the two-component regulatory system. Here, we first demonstrate the visualization of a phosphorylated-His protein in the HK reaction using Mn^{2+}–Phos-tag SDS-PAGE.

A recombinant histidine kinase ΔThkA, a deletion mutant (residue 408-755) of HK-TM1359 (pir id: H72262) from hyperthermophile *Thermotoga maritima*, was purified according to a previously described method [4]. The reaction pre-mixture (total volume of 0.20 ml) for the autophosphorylation assay was composed of 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 20 μM $MgCl_2$, and 5.6 μM ΔThkA. The reaction was initiated by the addition of 1/9th volume of 2.0 mM ATP at room temperature. After 0.5, 1, 2, 4, or 6 minutes, 6.0 μL of the reaction mixture was mixed with 10 μL of a stop solution composed of 0.21 M

Tris-HCl (pH 6.8), 13% (w/v) SDS, 32% (v/v) glycerol, 0.33 M DTT, 0.05% (w/v) bromophenol blue, and 1.0 mM EDTA at 70 °C.

SDS-PAGE was performed using a 1-mm-thick, 8.5-cm-wide, 9.5-cm-long gel at 120 V and room temperature according to a previously described method [1]. The gel consisted of 1.8 mL of a stacking gel (3.0% (w/v) acrylamide, 0.12 M Tris-HCl (pH 6.8), and 0.20 % (w/v) SDS) and 6.3 mL of a separating gel (7.5% (w/v) acrylamide, 0.37 M Tris-HCl (pH 8.8), 0.20% (w/v) SDS, 0.10 mM acrylamide-pendant Phos-tag, and 0.20 mM $MnCl_2$). The electrophoresis running buffer consisted of 25 mM Tris, 192 mM glycine, and 0.10% (w/v) SDS. To calibrate the amount of protein, 8, 16, 24, 32, and 40 pmol of ΔThkA, which had been treated with the stop solution in the same manner as shown above, were loaded on the gel (calibration lanes in Fig. 1A). Each of the assayed samples (16 μL, total 30 pmol of ΔThkA) was loaded on the same gel (assay lanes in Fig. 1A). After the electrophoresis, the gels were soaked in a breaching solution (30% methanol and 10% acetic acid) for 10 min. The gels were stained in a CBB solution, prepared by adding one tablet of PhastGel® Blue R (GE Healthcare) in 400 mL of breaching solution, for 30 min, and then washed with the breaching solution until the background of the gel was completely de-colored. The intensity of the protein band in the gel was analyzed with the Image J program (free share soft, available on http://rsb.info.nih.gov/ij/). An autoradiography assay using [γ-^{32}P]-ATP (0.09 MBq/nmol) was performed according to a previously described method [5].

As shown in the calibration lanes of Fig. 1A, the ATP-untreated (nonphosphorylated) ΔThkA showed a single band, whereas the ATP-treated ΔThkA gave two migration bands in the assay lanes. The lower bands appeared at the same position (relative mobility, $R_f = 0.34$) as that for the nonphosphorylated HK, where $R_f = 1$ is the mobility of BPB. The upper bands at $R_f = 0.29$ in the assay lanes are assigned to phosphorylated ΔThkA, because the position is consistent with that observed by ^{32}P autoradiography (see Fig. 1A). The difference in mobility between the upper and lower bands shows that the phosphorylated ΔThkA was preferentially captured by the Mn^{2+}–Phos-tag in the gel. This behavior of the phosphorylated HK is comparable to that of phosphorylated proteins containing phospho-Ser,

phospho-Thr, and phospho-Tyr residues in a previous report [1].

The gel image of the calibration lanes in Fig. 1A gave a linear relationship between the band intensity (AU) and amount of ΔThkA (pmol) (see Fig. 1B). The amount of the phosphorylated and nonphosphorylated proteins could then be estimated on the basis of the upper and lower bands in the assay lanes of Fig.1A, and were plotted against reaction time in Fig. 1C. The amount of phosphorylated ΔThkA produced increased time-dependently, consistent with the result from ^{32}P radioisotope method, while the total amount of nonphosphorylated and phosphorylated ΔThkA in each lane was nearly constant. This technique can be quantitatively useful in assays of HK reactions in the two-component regulatory system.

In summary, Mn^{2+}–Phos-tag can be useful for capturing phosphorylated His (N-phosphate) in protein samples, as well as O-phosphates. We believe that phosphoproteomics on HK would progress greatly by the effective and quantitative separation of phosphorylated HK using Mn^{2+}–Phos-tag SDS-PAGE.

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Figure legends

Fig. 1. Mn^{2+}–Phos-tag SDS-PAGE of ΔThkA. (A) Autophosphorylation assay of ΔThkA in a polyacrylamide gel (7.5% acrylamide, 100 μM Mn^{2+}-Phos-tag). The native ΔThkA (Calibration lanes: 8, 16, 24, 32, 40 pmol) and ATP-treated ΔThkA (Assay lanes: total 30 pmol) were loaded on the same gel, as shown in the left panel. Autoradiography under the same conditions using ^{32}P is shown in the right panel. Open and closed arrowheads indicate nonphosphorylated and phosphorylated ΔThkA, respectively. (B) Linear relationship between band intensity (AU) and the amount of ΔThkA (pmol). Each plot is the mean value of triplicate experiments. Solid line is the linear fitting of the data. (C) Autophosphorylation activity of ΔThkA. Open circles with solid line, phosphorylated ΔThkA; squares with dotted line, nonphosphorylated ΔThkA; triangles, sum of nonphosphorylated and phosphorylated ΔThkA; closed circles with dashed line, phosphorylated ΔThkA determined using ^{32}P. Each symbol is the mean value of triplicate experiments.

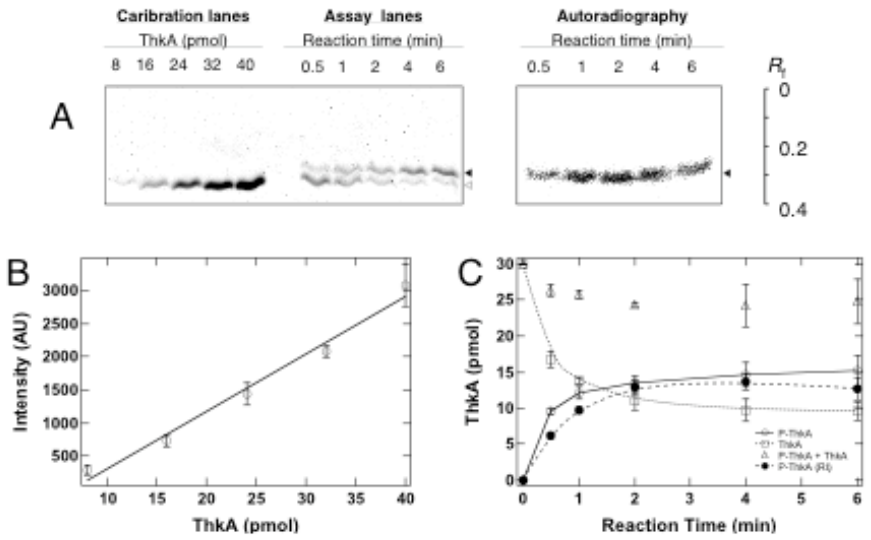

Fig. 1 Yamada et al.

Fig. 1