# Protein histidine phosphatase activity in rat liver and spinach leaves

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**Abstract Whole cell extracts from rat liver or spinach leaves contain divalent ion-independent protein histidine phosphatase activity due to phosphatases of the PP1/PP2A family. In the rat liver extract, almost all the activity was found in the PP1, $PP2A_1$ and $PP2A_2$ peaks. In the spinach leaf extract, four phosphorylase phosphatase activity peaks were resolved — three containing PP1 and one containing PP2A — and all showed histidine phosphatase activity. Thus, protein histidine phosphatase activity is expressed in the cytosolic forms of protein phosphatases of the PP1/PP2A family in mammalian and plant cells.**

*Key words:* Phosphoprotein phosphatase; Protein histidine phosphatase; Phosphohistidine; Rat; Spinach

## 1. Introduction

Protein phosphorylation is a pervasive signaling mechanism in both eukaryotes and prokaryotes. Direct activation of protein kinase receptors or activation of protein kinases by second messengers followed by cascades of protein kinases and phosphatases are used by cells to detect extracellular signals and transduce them into cellular responses. In prokaryotes, the two-component regulatory system works through the phosphorylation of a histidine residue and an aspartate residue [1,2]. Two eukaryotic genes with open reading frames homologous to the sequences that code for bacterial histidine kinases have recently been discovered [3–5] and a mitochondrial protein serine kinase homologous to bacterial histidine kinases has been isolated [6]. However, almost all other studies in eukaryotes have focused on the phosphorylation of serine, threonine and tyrosine residues since partial acid hydrolysis, as used for conventional phosphoamino acid analysis, destroys the phosphoramidate bonds found in phosphohistidine, phospholysine or phosphoarginine [7]. Phosphoramidate bonds and protein kinases that phosphorylate arginine, histidine or lysine have been reported in eukaryotes [8–25] and reviewed recently [26]. *Saccharomyces cerevisiae* contains a kinase that transfers phosphate from ATP to histidine-75 in the protein, histone H4, in vitro [27]. Similar enzymes have been reported in rat tissues and cell cultures and in nuclei from *Physarum* [9,10,28]. The yeast protein histidine kinase has been purified and is a protein of molecular weight 31,000 [15]. Its phosphorylation reaction is very specific for histidine residue 75 in histone H4 or peptides of the same sequence [27].

The family of known protein serine/threonine phosphatases is growing [29,30] but cellular protein serine/threonine phosphatase activity is dominated by protein phosphatases 1, 2A, 2B (calcineurin) and 2C [31,32]. Protein phosphatases 1 and 2A are divalent ion-independent while PP2B requires calcium and PP2C requires magnesium. In the cell, the catalytic subunits of PP1 and PP2A are complexed with other proteins to form heteromeric dimer or trimer complexes. Complex formation affects the activity, folding, targeting and substrate preferences of PP1 and PP2A [32–34]. Protein histidine phosphatases have not been described in prokaryotes (except for that coded by phage $\lambda$ [35–37]) as the phosphate from phosphohistidine is transferred to aspartate in a phosphotransfer reaction that is not regarded as a phosphatase activity [2]. Similarly, in the phosphoenolpyruvate-sugar transport system in prokaryotes, phosphohistidine is dephosphorylated in phosphotransfer reactions rather than protein phosphatase reactions [38]. In eukaryotes, Ohmori et al. [39] isolated two lysine/histidine phosphatases that may be protein lysine/histidine phosphatases and phosphatases that dephosphorylate polymers of phosphohistidine or phospholysine have been described [40]. Neither PP2B nor protein tyrosine phosphatase PTP 1B displays protein histidine phosphatase activity towards histone H4 phosphorylated on His-75 by the yeast protein histidine kinase [41]. However, the catalytic subunits of PP1, PP2A and PP2C are excellent protein histidine phosphatases [41] as is the $\lambda$ phosphatase (Kim and Matthews, manuscript submitted for publication). In this paper we demonstrate that: (i) PP1 and PP2A act as protein histidine phosphatases in their heteromeric intracellular forms in both animal and plant cells; and (ii) they account for almost all cytosolic divalent ion-independent phosphatase activity towards histone H4 phosphorylated on His-75.

## 2. Materials and methods

### 2.1. Materials

Protein histidine kinase was purified from *Saccharomyces cerevisiae*, as described, including the chromatography on MonoS [15]. Histone H4 was isolated from total calf thymus histone (Sigma Chemical Co.) by chromatography on a large Bio-Gel P-10 column (5 cm diameter × 1 m long) in 10 mM HCl. Protein phosphatases and inhibitors were generous gifts from Dr. Philip Cohen and his colleagues. The catalytic subunits of PP1 and PP2A were purified from rabbit skeletal muscle by Dr. D. Schelling [42]. Okadaic acid was a generous gift from Dr. Y. Tsukitani, Fujisawa Pharmaceutical Co., Tokyo, Japan. Inhibitor-2 expressed in *E. coli* was a gift to Dr. Philip Cohen from Dr. Anna De Paoli-Roach [43]. [$^{32}$P]Glycogen phosphorylase *a* was prepared by Dr. R. MacKintosh [44].

### 2.2. Extracts

Rat liver extracts were prepared by Mr. P. Ferrigno from homogenates made as described previously [45,46]. Briefly, finely chopped livers were homogenized in 3 volumes of ice-cold buffer (2 mM Na-EGTA, 2 mM Na-EDTA, 250 mM sucrose, 0.1% 2-mercaptoethanol supplemented with 0.1 mM PMSF and 1 mM benzamidine) using a Potter-Elvehjem homogenizer. The homogenate was clarified by centrifugation at 10,000 × *g* for 10 min at 4°C and the supernatant decanted and recentrifuged for 60 min at 100,000 × *g*.

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Spinach leaves were homogenized in 2 volumes of 50 mM Tris-HCl, pH 7.8 (4°C), 0.1 mM EDTA, 10% (v/v) glycerol, 1 mM benzamidine, 1 mM PMSF, 0.1% (v/v) 2-mercaptoethanol. The homogenate was filtered through cheesecloth, centrifuged at 15,000 × *g* for 5 min and 4 ml of this extract was diluted into 10 ml buffer A (20 mM triethylamine-HCl, pH 7.5, 0.1 mM Na-EGTA, 5% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol).

*2.3. Chromatography*

0.5 ml of extract was diluted into 10 ml buffer A and loaded onto an analytical (1 ml) MonoQ column previously equilibrated with buffer A and run at 1 ml/min. The column was washed for 10 min with buffer A and then eluted with a linear gradient from buffer A to 0.5 M NaCl in buffer A over 40 min followed by an increase to 1 M NaCl in buffer A over 5 min [47]. The first 20 ml of eluent was collected as one fraction — it did not contain phosphatase activity. The remaining 45 ml was collected into 90 fractions of 0.5 ml. For most experiments, each fraction was collected in 0.5 ml glycerol, mixed immediately after elution from the column and stored at −20°C.

*2.4. Phosphatase assays*

Protein histidine phosphatase activity was assayed with histone H4 as substrate as described [48] while protein serine phosphatase activity was measured with phosphorylase [44]. For experiments with Inhibitor-2, the enzyme was pre-incubated with the inhibitor for 10 min before adding substrate. For both assays, 1 Unit of activity corresponds to the release of 1 μmol of phosphate per min.

## 3. Results and discussion

Protein histidine phosphatase measurements were made on a rat liver cytosolic extract in which a 1:32,000-fold dilution (final protein concentration 1 μg/ml) gave 4 μUnits/ml of histidine phosphatase activity, in the absence of added divalent ions. More than 90% of this activity was lost when 330 nM okadaic acid was included. This shows that most, or all, of the divalent ion-independent protein histidine phosphatase activity in the extract is due to protein phosphatases of the PP1/PP2A family and that the release of $^{32}$P-radioactivity from histone H4 was not due to proteolysis. Fifty one per cent of the activity was inhibited by 3 nM okadaic acid, a concentration which inhibits PP2A completely but hardly affects PP1 [49,50].

A sample of the extract was subjected to ion exchange chromatography on a MonoQ column which separates the phosphorylase phosphatase activity into 3 peaks [45,47]. The first peak comprises PP1 because it is inhibited by Inhibitor-2 and 1 μM okadaic acid and is unaffected by 3 nM okadaic acid. The two later-eluting peaks contain two forms of PP2A and are inhibited completely by 3 nM okadaic acid and are unaffected by Inhibitor-2. $PP2A_1$ contains subunits A, B and C, and $PP2A_2$ contains subunits A and C [47]. The histidine phosphatase activity also showed 3 major peaks at the identical positions to the 3 major peaks of phosphorylase phosphatase activity (Fig. 1).

A separate minor peak of histidine phosphatase activity eluted at 9 ml, well before the PP1 peak, although no phosphorylase phosphatase activity was detected before the PP1 peak (Fig. 1). The peak at 9 ml was inhibited by 3 nM okadaic acid, indicating a member of the PP2A family of protein phosphatases. Although minor, quantitatively, this peak is of interest because its phosphatase activity is relatively specific for phosphohistidine, unlike the major isotype of PP2A.

The histidine phosphatase activity of the first major peak was insensitive to 3 nM okadaic acid but completely inactivated by 1 μM okadaic acid, showing that the major forms of PP1 in rat liver extracts dephosphorylate phosphohistidine.

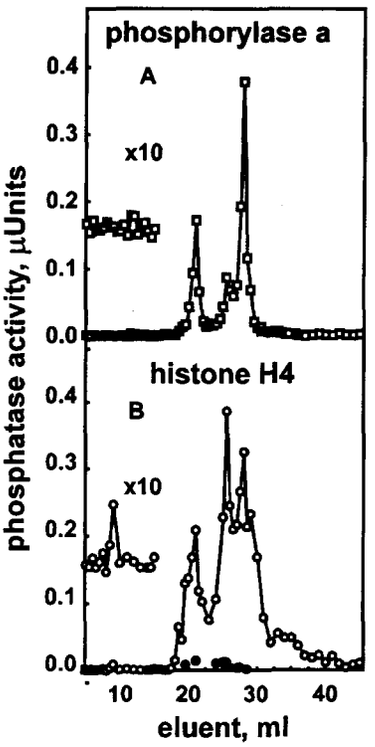


Fig. 1. Protein phosphatase activity in rat liver cytosol. A rat liver extract was fractionated by chromatography on a MonoQ column and the fractions assayed for phosphorylase *a* phosphatase activity (panel A) or histidine phosphatase activity (panel B). The insets show the same data plotted on a 10-fold more sensitive scale. In both panels, the open symbols show data obtained in the absence of inhibitors; the filled symbols in panel B show data obtained in the presence of 1 μM okadaic acid.

The first peak of PP2A had a much higher histidine phosphatase:serine phosphatase activity ratio than the second peak. The histidine phosphatase activity of the peak associated with $PP2A_1$ was only partly sensitive to 3 nM okadaic acid but completely inhibited with 1 μM okadaic acid. The catalytic subunit, $PP2A_C$, is fully inhibited by 3 nM okadaic acid when either phosphohistidine in H4 or phosphorylase is the substrate [41]. Thus, the $PP2A_1$ peak contains some histidine phosphatase activity that behaves like $PP2A_1$ and some activity that behaves differently and needs further characterization. Nearly all the histidine phosphatase activity associated with the second PP2A peak was sensitive to 3 nM okadaic acid consistent with its identity as $PP2A_2$.

In order to determine whether PP1 and PP2A were also the major phosphatases in plant cells, similar experiments were carried out with spinach leaf extracts. Four peaks of phosphorylase phosphatase activity were detected (Fig. 2A). The last peak dephosphorylated HMGI phosphorylated by $p34^{cdc2}$ [45], was resistant to 200 nM Inhibitor-2 (Fig. 2C) and was sensitive to 2 nM okadaic acid (Fig. 2D), indicating that it is a PP2A-like phosphatase. The first 3 peaks were resistant to 2 nM okadaic acid but largely inhibited by 200 nM Inhibitor-2 and 100 nM okadaic acid and they could not dephosphorylate HMGI. Thus they contain a PP1-like phosphatase.

Histidine phosphatase activity was measured on a similar preparation (Fig. 2B). Each of the 4 peaks of phosphorylase

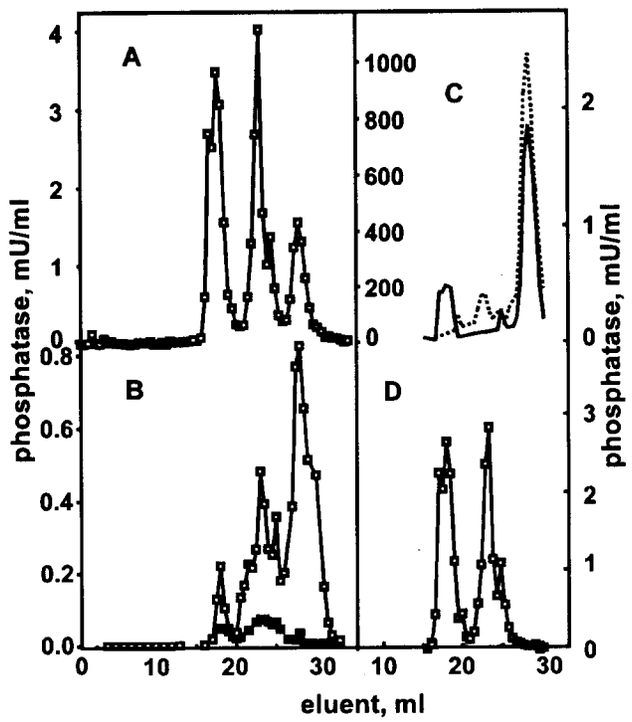


Fig. 2. Protein phosphatase activity in spinach leaf cytosol. A spinach leaf extract was fractionated by MonoQ column chromatography and the fractions assayed for protein phosphatase activity. The left-hand axis in panel C is the radioactivity released from HMGI in the phosphatase assay. The other vertical axes show phosphatase activity in mUnits/ml. The individual data points are omitted in panel C, for clarity, although the lines are drawn through the points as in the other panels. Panel A: phosphorylase *a* activity. Panel B: histidine activity alone (open symbols) or with 100 nM okadaic acid (filled symbols). Panel C: phosphorylase *a* activity in the presence of 200 nM Inhibitor-2 (solid line); HMGI activity (dotted line). Panel D: phosphorylase phosphatase activity in the presence of 2 nM okadaic acid.

phosphatase activity also showed histidine phosphatase activity. The fourth peak of phosphorylase phosphatase activity showed, relatively speaking, much more histidine phosphatase activity than the other peaks. This is comparable with the behavior of the $PP2A_1$ peak from rat liver. The histidine phosphatase activity of the last peak was inhibited by 100 nM okadaic acid (Fig. 2B), as expected for a $PP2A_1$-like enzyme. The earlier peaks were substantially, but not completely inhibited by 100 nM okadaic acid, consistent with their containing PP1-like phosphatases. Thus, in spinach as in rat liver all the phosphorylase phosphatase activities also show histidine phosphatase activity.

Each of the peaks of phosphorylase phosphatase resolved by MonoQ chromatography of rat liver and spinach leaf extracts also shows substantial histidine phosphatase activity. Since the histone H4 substrate is phosphorylated on histidine, rather than serine, phosphorylated in phosphorylase *a*, the cellular forms of PP1 and PP2A are protein histidine phosphatases as well as protein serine phosphatases, like the isolated catalytic subunits [41]. Importantly, essentially all the histidine phosphatase activity is inhibited by okadaic acid, showing that all the histidine phosphatase activity in mammalian and plant extracts is catalyzed by members of the PP1/PP2A family of protein phosphatases.

It is now clear that eukaryotic cells contain extensive protein histidine phosphatase activity. Eukaryotic protein histidine kinases have been described, including mammalian, plant, yeast and slime mold [9,10,27,28], and in some circumstances, histidine phosphorylation is correlated with cessation of cell proliferation and commencement of differentiation [26]. The high activity of cellular protein histidine phosphatases is consistent with an important role for phosphohistidine in eukaryotes.

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