Partial Purification and Characterization of an Enzyme from Pea Nuclei with Protein Tyrosine Phosphatase Activity

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A pea (Pisum sativum L.) nuclear enzyme with protein tyrosine phosphatase activity has been partially purified and characterized. The enzyme has a molecular mass of 90 kD as judged by molecular sieve column chromatography and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Like animal protein tyrosine phosphatases, it is also inhibited by heparin and spermine but not by the acid phosphatase inhibitors citrate and tartrate or the protein serine/threonine phosphatase inhibitor okadaic acid. It dephosphorylates peptides tested but not the phosphoserine/threonine residues on casein and histone. Like some animal protein tyrosine phosphatases, it has a variable pH optimum depending on the substrate used: the optimum is 5.5 when the substrate is [32P]tyrosine-labeled lysozyme, but it is 7.0 when the substrate is [32P]tyrosine-labeled poly(glutamic acid, tyrosine). It has a K_m of 4 μM when the lysozyme protein is used as a substrate.

Protein phosphorylation/dephosphorylation is thought to be one of the major cellular regulatory mechanisms in plant cells (Ranjeva and Boudet, 1987; Roux, 1993). For example, in peas (Pisum sativum L.) the phosphorylation of DNA-binding factors appears to be involved in transcriptional regulation (Datta and Cashmore, 1989), and in many higher plants the activity of several key metabolic enzymes changes with their phosphorylation state (Budde and Chollet, 1988). There is a long-established literature concerning protein kinases in both plant and animal cells, but only in recent years has information regarding protein phosphatases also become extensive. Whereas much information is available about the structure, chemical properties, mechanism of action, and regulation of protein phosphatases in animal cells (Shenolikar and Nairn, 1991; Charbonneau and Tonks, 1992; Walton and Dixon, 1993), there is much less known about these enzymes and their functions in plant cells, despite some recent progress in this field. Several Ser/Thr protein phosphatases have been reported in plant cells (Polya and Haritou, 1988; Mackintosh et al., 1991; Sheen, 1993). The activities of protein Tyr kinase and phosphatase enzymes and some phosphotyrosine protein substrates they have been detected in plant cells (Elliott and Geytenbeek, 1985; Torruella et al., 1985; Cheng and Tao, 1989; Aggarwal et al., 1993). Whether the protein Tyr phosphorylation/dephosphorylation has any physiological function is not yet established.

While studying protein kinases in pea nuclei (Li et al., 1991; Li and Roux, 1992), we found both protein Ser/Thr phosphatase and PTPase activity in crude nuclear extracts. As part of our effort to understand the role of protein phosphorylation/dephosphorylation in plant nuclear metabolism, we have begun to investigate the properties of these protein phosphatases. Here we report the partial purification and characterization of a pea nuclear enzyme with PTPase activity.

MATERIALS AND METHODS

Plant Growth

Seedlings of pea (Pisum sativum L. cv Alaska) were grown in the dark for 7 d at 23 ± 3°C.

Chemicals

Aprotinin (bovine lung, in solution of approximately 1.4 mg/mL), heparin (porcine intestinal mucosa), okadaic acid, Percoll, PMSF, pNPP, poly(Glu,Tyr) (4:1), and spermine were from Sigma. The BCIP/NBT phosphatase substrate was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD), and c-src (p60c-src) kinase was from Upstate Biotechnology, Inc. (Lake Placid, NY). All buffers were prepared with water purified by a MilliQ water purification system (Millipore Corp., Bedford, MA).

Nuclei and Chromatin Fraction Preparations

All procedures were carried out at 4°C, except the HPLCs, which were run at room temperature (22 ± 3°C). Nuclei were isolated from the plumules of dark-grown pea seedlings as previously described by Datta et al. (1985). Routinely, the nuclei isolated from 50 to 60 g of pea plumes were used as the starting material. The nuclei were lysed by incubating them in buffer A (50 mM Hepes [pH 7.5], 1 mM EDTA, 0.5% [v/v] Triton X-100, 0.5 mM PMSF,

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; NBT, nitroblue tetrazolium; ENZYMASS, GluAsnAspTyrIle-AsnAlaSerLeu; MBP, myelin basic protein; pNPP, para-nitrophenyl phosphate; PTPase, protein Tyr phosphatase; RCML, reduced, carboxamidomethylated, and maleylated lysozyme.

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0.5% [v/v] aprotinin, 10 mM DTT) for 10 min at 4°C. The extract was centrifuged at 9000g for 1 min. The pellets obtained were designated as the chromatin fraction.

Purification of the Phosphatase

The chromatin fraction was extracted by stirring it for 20 min in 1.5 mL of buffer B (60 mM Hepes [pH 7.5], 3 mM MgCl$_2$, 0.3 mM NaCl, 0.5 mM PMSF, 0.5% [v/v] aprotinin, 14 mM 2-mercaptoethanol) and then centrifuging it at 9000g for 10 min. The supernatant, referred to below as the 0.3 mM NaCl extract, was carefully removed, and all of it was fractionated through a HPLC Ultraspherogel SEC 3000 molecular sieve column (7.5 mm × 30 cm; Beckman Instruments, Inc., Fullerton, CA) that had been previously equilibrated with buffer C (25 mM Tris-Cl [pH 7.5], 100 mM NaCl, 14 mM 2-mercaptoethanol). The active fractions from six runs were collected and combined and then concentrated by an Amicon Centricon-10 concentrator. This sample was applied to a HEMA IEC BIO DEAE 10 U column (7.5 mm × 7.5 cm; Alltech Associates, Inc., Deerfield, IL) that had been equilibrated with buffer D (25 mM Tris-Cl [pH 7.5], 14 mM 2-mercaptoethanol). The column was washed with buffer D. After unbound proteins were washed off the column, the bound proteins were eluted with buffer E (buffer D plus 0.9 mM NaCl). The active fractions were combined and used for the characterization studies of the PTPase activity unless otherwise indicated.

The enzyme samples used for detection of phosphatase activity after gel electrophoresis and for native molecular mass determination were purified by loading the 0.3 mM NaCl extract on the DEAE column without prior chromatography through the molecular sieve column. This enzyme preparation was designated DEAE-purified phosphatase.

Preparation of $^{32}$P-Labeled Substrates and Phosphatase Assays

Poly(Glu,Tyr) was phosphorylated in a reaction mixture (200 μL) that contained 50 mM Tris-Cl (pH 7.5), 10 mM MgCl$_2$, 0.1 mM sodium vanadate, 100 μM ATP, 12.5 μCi [γ-$^{32}$P]ATP, 0.6 mg poly(Glu,Tyr), and 15 units of c-src kinase. After incubation for 3 h at room temperature, the mixture was heated at 90°C for 5 min and subjected to a Bio-6 spin column (Bio-Rad) to resolve $^{32}$P-labeled-poly(Glu,Tyr) from unreacted ATP. The preparation then was dialyzed extensively against deionized H$_2$O. Histone (type III-S) was phosphorylated on Ser/Thr residues by a partially purified pea nuclear Ca$^{2+}$-dependent protein kinase (Li et al., 1991), and casein was phosphorylated on Ser/Thr residues by a pea nuclear type 2 casein kinase (Li and Roux, 1992) as described previously. Phosphorylated proteins were precipitated by 20% TCA and extensively dialyzed against deionized H$_2$O. [$^{32}$P]Tyrrlabeled RCML, [$^{32}$P]Tyr-labeled MBP, and [$^{32}$P]Tyr-labeled nonapeptide ENDYINASL were kindly provided by Dr. E. Fischer's laboratory (University of Washington, Seattle).

The protein phosphatase activity was determined by measuring the release of $^{32}$P from $^{32}$P-labeled substrates, according to the method of Tonks et al. (1988a, 1988b) with some modifications. For RCML as substrate, the basic reaction mixture contained 50 mM Mes-OH (pH 5.5), 1 μM RCML, 0.3 mg/mL BSA, 14 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, and the enzyme preparation in a final volume of 60 μL. The reaction was initiated by adding 20 μL of RCML substrate, which proceeded at 30°C for 15 min, and was stopped by the addition of 190 μL of 20% TCA and 10 μL of 25 mg/mL BSA as a carrier protein. The mixture was vortexed, frozen, and kept at −70°C for 30 min. The sample was thawed and centrifuged at 9000g for 5 min. A 200-μL aliquot of the supernatant was added to 2 mL of aqueous scintillant and counted in a Packard scintillation counter. Blank incubations were carried out in which the enzyme preparations were replaced by buffer D. One unit of enzyme activity was defined as the amount that released 1 pmol of phosphate/min. For reactions using poly(Glu,Tyr) as the substrate, 50 mM Mes-Oh (pH 5.5) was replaced by 50 mM Tris-Cl (pH 7.0). The reaction was initiated by adding 20 μL of substrate (3 × 10$^5$ cpm to 5 × 10$^4$ cpm), and the incubation time was 10 min at room temperature. The above assay conditions are referred to below as standard conditions for RCML and for poly-(Glu,Tyr). For other protein substrates, the assay method was essentially the same, with some minor modifications as described for the individual experiments. The phosphatase assay with ENDYINASL and MBP was carried out at pH 7.5 using an incubation time of 30 min in a room temperature, according to the method of Daum et al. (1993) with minor modifications.

The ability of the enzyme to use pNPP as a substrate was determined by assaying the release of p-nitrophenol from pNPP. The reaction mixture contained 50 mM Mes-Oh (pH 5.5) or 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 14 mM 2-mercaptoethanol, 10 mM pNPP, and the enzyme preparation in a total volume of 60 μL. The reaction mixture was incubated at 30°C for 30 min. The reaction was stopped by adding 60 μL of 2 N NaOH, and then the A$_{410}$ was measured in an ELISA plate reader. The amount of p-nitrophenol released was determined using the molar extinction coefficient of 17.5 × 10$^4$ M$^{-1}$ cm$^{-1}$.

Detection of Phosphatase Activity in Situ after Gel Electrophoresis

The phosphatase activity could be detected in gels after both SDS-PAGE (Laemmli, 1970) and native PAGE. The enzyme was renatured after SDS-PAGE, and the phosphatase activity was detected by using the BCP/NBT phosphatase substrate. The electrophoresis and renaturation procedures were accomplished according to the methods described by Guo and Roux (1990) with some modifications. Briefly, the enzyme samples were added to SDS sample buffer without boiling and electrophoresed on SDS-PAGE. The SDS was removed immediately after electrophoresis by washing the gel at room temperature with a buffer of 25 mM Tris-Cl (pH 7.5), 100 mM NaCl, 0.015% (v/v) Triton X-100 for 30 min with three changes of the buffer. The enzyme was allowed to renature in 25 mM Tris-Cl (pH 7.5), 0.5 mM DTT overnight at 4°C or at room temperature.
temperature for 4 to 5 h. The gel was washed twice with 100 mM Tris-Cl (pH 7.5) for 10 min and then incubated at room temperature in a reaction mixture containing 10 mL of 100 mM Tris-Cl (pH 7.5) and 1 mL each of BCIP and NBT. Any protein band with phosphatase activity was detected, usually within 4 or 5 h, by the purple color produced in situ. The reaction was terminated by washing the gel extensively with deionized H₂O. The method for detecting phosphatase activity after native gel electrophoresis was essentially the same as above, except that the steps for removal of SDS and renaturation of the activity in the gel were omitted. The electrophoresis was carried out using 7% acrylamide, according to the method described by Laemmli (1970), except that SDS was omitted from all components of the system.

The BCIP/NBT phosphatase activity could also be detected on a nitrocellulose membrane after transfer from an IEF agarose gel. The IEF electrophoresis and transfer methods were done according to the method of Guo and Roux (1990). The DEAE-purified phosphatase sample was concentrated by Centricon-10 and subjected to IEF on an analytical IEF gel (pH range 3-10, IsoGel; FMC Bio Product, Rockland, ME). When the focusing was complete, the gel slab was cut into several lanes. The proteins from one lane were transferred to a nitrocellulose membrane. The membrane was washed briefly with 100 mM Tris-Cl (pH 7.5), and then the color reaction was developed as described above for the gel assay. The other two lanes were each cut horizontally into 2.5-mm slices. Each slice was homogenized and extracted with 100 mM Tris-Cl (pH 7.5), 14 mM 2-mercaptoethanol, 1 mM EDTA. The extract was assayed for protein phosphatase activity using 3²P-labeled poly-(Glu,Tyr) as the substrate under the standard conditions, except that 0.5% (v/v) aprotinin and 10 μg/mL leupeptin were included in the reaction mixture, and the incubation time was 1 h at room temperature.

**Determination of Native Molecular Mass of the Phosphatase**

The native molecular mass of the phosphatase was determined on an HPLC molecular sieve column (G3000 SW; 30 cm × 7.8 mm; Supelco). A concentrated DEAE-purified sample was loaded onto the column, which was both equilibrated and eluted with a buffer containing 25 mM Tris-Cl (pH 7.0), 200 mM NaCl, 3 mM EDTA. The flow rate was 0.5 mL/min, and 0.25-mL fractions were collected. The estimate of the native molecular mass of the enzyme was based on comparison of retention times between active fractions and molecular mass standards.

**Protein Determination**

Protein concentration was determined with a Bio-Rad protein assay kit using BSA as a standard.

**RESULTS**

**Purification of the Phosphatase**

Pea nuclei purified by the method described were free from other intact organelles as judged by light and electron microscopic examination (Datta et al., 1985), and were more than 85% pure as judged by assays of enzyme markers for Golgi, ER, and mitochondria (Clark et al., 1993). After the nuclei were extracted with Triton X-100 and EDTA, the remaining chromatin fraction contained nuclear scaffolding proteins in addition to the chromatin, but it was essentially free of the nuclear envelope and other membranes, as reported by Li and Roux (1992).

The purification achieved at each step in the isolation procedure is presented in Table I. The enzyme was purified 51-fold with a final specific activity of 967 pmol min⁻¹ mg⁻¹ when using RCML as substrate. The elution profiles from the two column chromatographic steps used are shown in Figure 1. The most active fraction pooled from the molecular sieve column contained both a PTPase (Fig. 1A) and a protein Ser/Thr phosphatase activity (data not shown). The enzymes containing these two phosphatase activities could be separated by the DEAE column, with the one having Tyr phosphatase activity emerging in the flow-through fractions (Fig. 1B). The protein Ser/Thr phosphatase bound tightly to the column and was eluted by 0.9 M NaCl. It did not dephosphorylate [³²P]RCML but could dephosphorylate both ³²P-labeled histone and casein that had been labeled at Ser and Thr residues by a pea nuclear Ca²⁺-dependent protein kinase and a pea nuclear type 2 casein kinase, respectively (Li et al., 1991; Li and Roux, 1992) (data not shown). When the flow-through fraction of DEAE-purified phosphatase (containing PTPase activity) was analyzed by molecular sieve chromatography, its elution position from the column indicated a native molecular mass of approximately 90 kD (Fig. 1C).

The protein heterogeneity of the purest fraction eluting from the second (DEAE) column chromatography step was judged by the Coomassie blue-staining pattern after SDS-PAGE. The preparation contained two visible bands at 97 and 90 kD, with the 97-kD band being the more intensely staining one (Fig. 2A).

<table>
<thead>
<tr>
<th>Table 1. Purification of the pea nuclear phosphatase from nuclei of 60 g of pea plumules</th>
</tr>
</thead>
<tbody>
<tr>
<td>The enzyme was assayed using RCML as substrate.</td>
</tr>
<tr>
<td>Step</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>Chromatin extract</td>
</tr>
<tr>
<td>Molecular sieve column</td>
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<tr>
<td>DEAE column</td>
</tr>
</tbody>
</table>

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Figure 1. Elution profiles of A$_{280}$ (OD 280) and PTPase activity from the Ultraspherogel SEC3000 molecular sieve (A), DEAE (B), and TSK gel G3000 SW molecular sieve columns (C). The enzyme activity was assayed using RCML (A and B) or poly(Glu,Tyr) (C) as substrates. The molecular mass markers used to calibrate the column in C were: thyroglobulin (670 kD), ferritin (440 kD), catalase (232 kD), BSA (68 kD), ovalbumin (44 kD), and Cyt c (12 kD). Each point represents the mean of duplicate assays obtained under standard conditions. V$_0$, Void volume.

Determination of Phosphatase Activity after Electrophoresis

Since the final enzyme preparation was not homogeneous, gel assay methods were devised and used to identify the protein band(s) responsible for the phosphatase activity. Samples purified only through a molecular sieve column or only through a DEAE column showed phosphatase activity in gels after SDS-PAGE. Samples purified through both molecular sieve and DEAE columns were very dilute and somewhat less stable, and these samples typically did not show phosphatase activity in gels after SDS-PAGE but showed activity after native gel electrophoresis.

An enzyme sample concentrated from the active fractions of the molecular sieve column, fractionated by SDS-PAGE, and renatured in the gel contained two protein bands at 126 and 90 kD that showed BCIP/NBT phosphatase activity (Fig. 2B, lane 1). When the 0.3 M NaCl crude extract of nuclear proteins was purified directly by DEAE column chromatography and the fractions were assayed for phosphatase activity after SDS-PAGE, a 90-kD phosphatase in the flow-through fraction and a 126-kD phosphatase in the salt-eluted fraction were detected (Fig. 2B, lanes 2 and 3). Because the flow-through fraction contained PTPase but no protein Ser/Thr phosphatase activity and the bound fraction contained protein Ser/Thr phosphatase but no PTPase activity, we judge that the 90-kD protein band was responsible for PTPase activity and the 126-kD band may account for the Ser/Thr phosphatase activity. In support of this conclusion was the finding that...
Substrate Specificity and Dephosphorylation of \[^{32}\text{P}]\text{RCML}\) and \[^{32}\text{P}]\text{Poly(Glu,Tyr)}\)

Among the substrates tested, the 90-kD pea nuclear phosphatase could dephosphorylate \[^{32}\text{P}]\text{Tyr}\)-labeled RCML, poly(Glu,Tyr) (Fig. 4), MBP, and ENDOYINASL. Dephosphorylation of MBP and ENDOYINASL was 59 and 20%, respectively, but under the same reaction conditions the dephosphorylations of \[^{32}\text{P}]\text{Ser/Thr}\)-labeled casein and histone were both less than 2%. In a positive control experiment carried out at the same time, the \[^{32}\text{P}]\text{Poly(Glu,Tyr)}\) as substrate, all three bands had activity (Fig. 3). This result indicates that the same enzyme is responsible for both BCIP/NBT and PTPase activity.

Figure 3. Detection of phosphatase activity and pi determination after IEF gel electrophoresis. After the enzyme samples were subjected to IEF, the gel was cut into several lanes, one lane of separated proteins was transferred onto a nitrocellulose membrane, and BCIP/NBT phosphatase activity was detected on the membrane (inset). The other two lanes of focused gel were cut into 2.5-mm slices. The proteins extracted from each slice were tested for PTPase activity using \[^{32}\text{P}]\text{Poly(Glu,Tyr)}\) as substrate. The pi markers are indicated under the inset, and the pIs of active bands are indicated by asterisks above the inset.

Figure 4. Time course of dephosphorylation of RCML and poly(Glu,Tyr) by the purified protein phosphatase. The PTPase activity was measured under the standard conditions at different time intervals as indicated. Each point represents the mean of duplicate assays. The inset shows an autoradiogram of the gel after SDS-PAGE of RCML samples taken at different time intervals after initiation of the dephosphorylation reaction. Loss of \(^{32}\text{P}\) label from samples was determined by measuring the decrease in densities of the bands using a FB910 densitometer (Fisher Scientific).

The 90-kD enzyme with PTPase activity was further characterized using \[^{32}\text{P}]\text{RCML}\) and \[^{32}\text{P}]\text{Poly(Glu,Tyr)}\) as substrates. Under the conditions used, the dephosphorylation of RCML was linear up to 20 min with about 30% release of total \(^{32}\text{P}\) (Fig. 4). The dephosphorylation was also monitored by stopping the reaction at different time intervals and then assaying the amount of label remaining in RCML by autoradiography of the dried gel after SDS-PAGE (Fig. 4, inset). Dephosphorylation of RCML estimated by autoradiography was parallel with that determined by the standard assay. The RCML protein bands on both SDS-PAGE and the autoradiogram were very sharp even after a prolonged incubation time (up to 60 min), indicating that reduced radioactivity was not due to proteolytic cleavage of the lysozyme by protease activity. The apparent \(K_m\) value for the dephosphorylation of RCML was approximately 4 \(\mu\)M based on the initial velocity of the reactions at five different substrate concentrations. Dephosphorylation of poly(Glu,Tyr) was linear for up to 10 min, resulting in the release of 15% of the total \(^{32}\text{P}\) under standard assay conditions (Fig. 4). The pH optimum was 5.5 for RCML and 7.0 for poly(Glu,Tyr) under the conditions used (Fig. 5).

Effects of Various Agents on Phosphatase Activity

Various agents were tested for their effects on the PTPase activity (Fig. 5). The activity was strongly inhibited for
both substrates by micromolar concentrations of molybdate and vanadate, which are known as animal PTPase inhibitors (Chernoff and Li, 1983; Tonks et al., 1988b, 1991). Several PTPases are known to be strongly inhibited by Zn$^{2+}$ (Boivin et al., 1987; Cheng and Tao, 1989; Zhao et al., 1993), and the pea phosphatase was also inhibited by this cation but only when poly(Glu,Tyr) was used as substrate. Citrate and tartrate, both documented inhibitors of some acid phosphatases (Verjee, 1969), showed no inhibitory effects, with citrate actually having some stimulatory effect instead. Sodium fluoride, a strong inhibitor of Ser/Thr protein phosphatase activity (Lau et al., 1989), also showed some inhibitory effect on the activity of the 90-kD phosphatase when RCML was used as substrate but no obvious
effect on enzyme activity when poly(Glu,Tyr) was used as substrate. Among other agents tested, Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$ had no significant effect on the enzyme activity, whereas EDTA and EGTA showed stimulating effects, as has been reported for some animal PTPases (Chernoff and Li, 1983; Tung and Reed 1987; Tonks et al., 1991). Okadaic acid, a potent protein inhibitor of protein Ser/Thr phosphatase type 1 and type 2A (Cohen et al., 1990), had no effect on the pea nuclear phosphatase up to 1 μM, but spermine (Fig. 6A) and heparin (Fig. 6B) both inhibited the enzyme in a dose-dependent manner.

### Table II. Effects of various agents on the phosphatase activity

All effects were assayed under standard conditions, except that EDTA and EGTA were omitted from the basic reaction mixture to which the agents were added. The relative activity represents the mean of duplicate assays.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>Relative Activity %</th>
<th>RCML</th>
<th>Poly(Glu,Tyr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NH$_4$ molybdate</td>
<td>100 μM</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vanadate</td>
<td>100 μM</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>100 μM</td>
<td>56%</td>
<td>86%</td>
<td>1</td>
</tr>
<tr>
<td>NaF</td>
<td>5 mM</td>
<td>32%</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>1 mM</td>
<td>108%</td>
<td>87%</td>
<td></td>
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<tr>
<td>MgCl$_2$</td>
<td>5 mM</td>
<td>132%</td>
<td>92%</td>
<td></td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>1 mM</td>
<td>129%</td>
<td>95%</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>5 mM</td>
<td>236%</td>
<td>185%</td>
<td></td>
</tr>
<tr>
<td>Tartrate</td>
<td>5 mM</td>
<td>201%</td>
<td>194%</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM</td>
<td>259%</td>
<td>192%</td>
<td></td>
</tr>
<tr>
<td>EGTA</td>
<td>5 mM</td>
<td>220%</td>
<td>120%</td>
<td></td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>1 μM</td>
<td>96%</td>
<td>100%</td>
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</table>

**DISCUSSION**

The pea nuclear phosphatase described here has properties resembling those of many animal PTPases. The molecular masses of animal PTPases vary widely from 15 kD to more than 100 kD, including several in the 90- to 95-kD range (Lau et al., 1989; Charbonneau and Tonks, 1992). On the basis of our activity gel assays, we judge a 90-kD protein to be the one responsible for the PTPase activity detected in our purest fractions. The position of PTPase activity elution from the molecular sieve column is also consistent with a molecular mass assignment of approxi-
mately 90 kD for the enzyme; thus, this enzyme is apparently monomeric.

The artificial substrate BCIP/NBT was used to detect phosphatase activity both in gels after acrylamide gel electrophoresis and in the nitrocellulose membrane after IEF electrophoresis and transfer. The range of pIs detected (5.9-6.2) (Fig. 3) could indicate multiple modifications of one enzyme, since there was only one active band in the gel after SDS-PAGE (Fig. 2B, lane 2) and the linearity of the enzyme kinetics data would be more easily explained by there being only one phosphatase in the assay. An obvious modification to consider would be phosphorylation, which is known to change the pIs of proteins and to regulate the activity of phosphatases (Walton and Dixon, 1993).

The pIs of the phosphatase(s) were obtained using the BCIP/NBT detection technique, with the modification that the phosphatase activity was detected on the nitrocellulose membrane after IEF electrophoresis and transfer. The range of pIs detected (5.9-6.2) (Fig. 3) could indicate multiple phosphatases. More likely it was the result of posttranslational modifications of one enzyme, since there was only one active band in the gel after SDS-PAGE (Fig. 2B, lane 2) and the linearity of the enzyme kinetics data would be more easily explained by there being only one phosphatase in the assay. An obvious modification to consider would be phosphorylation, which is known to change the pIs of proteins and to regulate the activity of phosphatases (Walton and Dixon, 1993).

The BCIP/NBT phosphatase assay would detect the activity of acid and alkaline phosphatases as well as of PTPases. Our results indicate that the same enzyme that generated the colorimetric signal could also dephosphorylate [32P]poly(Glu,Tyr), and the pH optimum for this dephosphorylation was 7.0. This would suggest that this PTPase activity should not be attributed to an alkaline or acid phosphatase.

When protein/peptide substrates were tested, the activity of the 90-kD nuclear phosphatase showed significant specificity. The enzyme could dephosphorylate all four of the [32P]Tyr-labeled proteins and peptide tested but not [32P]Ser/Thr-labeled casein or histone.

The pea nuclear phosphatase also shares in common with animal PTPases the property of having a different pH optimum depending on the substrate presented. It has a pH optimum of 5.5 using RCML as a substrate, but its optimum pH shifts to 7.0 when poly(Glu,Tyr) is used as a substrate (Fig. 5). The pH optimum of animal PTPases varies with the isozyme form tested, the substrate used, and the assay conditions used (Lau et al., 1989). For example, human placental PTPase has a pH optimum of 5.5 when pNPP is used as substrate, but when RCML is used the pH optimum is shifted to 7.5 (Tonks et al., 1988b). Similar phenomena were found for other PTPases, such as protein Tyr phosphatase 1C (Zhao et al., 1993). These results indicate that we cannot be certain of the true pH optimum of the enzyme in vivo when physiological substrates are not used for the enzyme assay.

A number of chemical modulators have been widely used to study animal PTPases and to identify them diagnostically (Walton and Dixon, 1993). These agents were tested for their effects on the pea phosphatase. As shown in Table II, PTPase activity was almost totally blocked by 100 μM molybdate and vanadate, which are known as strong PTPase inhibitors. Zn2+ had little effect on phosphatase activity when RCML was used as a substrate, but it was strongly inhibitory when poly(Glu,Tyr) was used. Similarly, the Zn2+ sensitivity of some animal PTPases is substrate dependent (Boivin et al., 1987; Yang et al., 1993). In general, the effect of Zn2+ on animal PTPases varies, strongly inhibiting some of them (Tonks et al., 1988b; Zhao et al., 1993) and having little effect on others (Chernoff and Li, 1983; Tonks et al., 1991).

The pea nuclear phosphatase is inhibited by NaF when tested at its acidic pH optimum with RCML but not when tested at its neutral pH optimum with poly(Glu,Tyr). Although NaF is more characteristically an inhibitor of protein Ser/Thr phosphatases than of PTPases, several animal PTPases are known to be inhibited by NaF, especially when the enzymes are assayed at acidic pH conditions (Boivin et al., 1987). As is also true for some animal PTPases, the pea phosphatase was stimulated by EDTA, and to a lesser extent by EGTA, but was not affected significantly by Ca2+, Mg2+, or Mn2+ (Chernoff and Li, 1983; Tung and Reed, 1987; Tonks et al., 1991). Two known acid phosphatase inhibitors, citrate and tartrate, not only did not inhibit the pea enzyme but actually stimulated it about 2-fold (Table II). This result indicates that the mechanism of dephosphorylation of RCML by the pea protein phosphatase may differ from that of classical acid phosphatases, although its dephosphorylation of the protein substrate is optimal at acidic pH. Okadaic acid, a well-known inhibitor of protein Ser/Thr phosphatase type 1 and type 2A, has no effect on pea phosphatase activity.

Both polycations and polyanions affect the activity of some animal PTPases. Although most animal PTPases are inhibited by heparin (Tonks et al., 1988b, 1991; Zhao et al., 1993), the effect of spermine seems to vary among the different enzymes. Some are stimulated (Tonks et al., 1988b, 1991; Zhao et al., 1993), and some are inhibited (Boivin et al., 1987; Yang et al., 1993). The pea nuclear phosphatase is inhibited by both agents. A possible significance of nuclear phosphatase inhibition by spermine may be seen in the fact that spermine treatment stimulates the phosphorylation of several pea nuclear proteins (Datta et al., 1986), including a 77-kD protein that we have found that is recognized by anti-phosphotyrosine antibodies (data not shown). The possibility that the spermine inhibition of a phosphatase could be a means of stabilizing the spermine-induced phosphorylation state of nuclear proteins deserves further investigation.

All of the biochemical properties of the pea nuclear protein phosphatase reported here can be found among the animal PTPases. Although they are not conclusive, the assays conducted, when taken together, show that the pea nuclear phosphatase is more like a PTPase than like any other type of protein phosphatase characterized so far. The fact that the 90-kD nuclear phosphatase has a pH optimum of 5.5 for the modified lysozyme substrate might suggest that it should be classified as an acid phosphatase. How-
ever, even when assayed at acid pH, the pea phosphatase has very low activity toward pNPP, an artificial substrate standardly used to characterize acid phosphatases. Its specific acidic phosphatase activity toward pNPP (26 pmol min\(^{-1}\) mg\(^{-1}\)) is negligible compared with those of acid phosphatases isolated from wheat germ, which have a specific activity range from 420 to 520 mmol min\(^{-1}\) mg\(^{-1}\) (Verjee, 1969). Furthermore, the acid phosphatase inhibitors citrate and tartrate are ineffective in blocking the PTPase activity of the 90-kD pea enzyme. These results are inconsistent with the interpretation that the nuclear phosphatase is a generic acid phosphatase.

Evidence that PTPase enzymes exist in plant cells is beginning to accumulate. Cheng and Tao (1989) purified a PTPase-like enzyme from wheat seedlings with biochemical properties similar to animal PTPases. Like the pea nuclear phosphatase described here, this enzyme showed a high degree of specificity toward phosphotyrosine peptides and was inactive toward phosphoserine/threonine proteins at neutral pH. It, too, was clearly distinguishable from plant acid phosphatases. Unlike the pea phosphatase, it had a relatively small molecular mass (35 kD), and it was not reported to be associated with any cellular organelle.

Aggarwal et al. (1993) found that Rubisco from *Cicer* is phosphorylated at Tyr residues and can be dephosphorylated by the protein phosphatase described by Cheng and Tao (1989). The activity of Rubisco seems to be regulated by the protein phosphatase described by Cheng and Tao (1989). The data presented here concerning the 90-kD protein phosphatase are consistent with the interpretation that it is a PTPase and to clarify its possible roles in cells.

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