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Purification and characterization of a protein phosphatase from winged bean

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Abstract

A protein phosphatase (WbPP) has been purified from the soluble fraction of the winged bean (*Psophocarpus tetragonolobus*) shoot extract. The preparation is essentially homogenous as shown by the constant specific activity of the enzyme across the peak fractions, eluted from the thiophosphorylated histone-Sepharose affinity column, the last step of purification and by single protein bands on polyacrylamide gel electrophoresis (PAGE) in the presence as well as absence of denaturating agents. The monomeric nature of WbPP is revealed by an M_r of 92 000 and 85 000, respectively, as estimated by SDS-PAGE and gel permeation chromatography under non-denaturating conditions. Autophosphorylated calmodulin-like domain protein kinase (P-WbCDPKI) [Saha, P., & Singh, M. (1995). *Biochem. J.*, *305*, 205] and phosphohistone H1 (P-hisH1), prepared by using the other homologous CDPK, i.e. WbCDPKII [Ganguly, S., & Singh, M. (1998). *Phytochemistry*, *48*(1), 61], are good substrates of the purified enzyme, while P-hisH1 and phosphocasein prepared by using heterologous cAMP-dependent protein kinase, are respectively very poor and totally inactive as substrate. WbPP is adjudged to be a protein phosphoserine phosphatase since phosphoserine is the only phosphorylated amino acid residue detected in our earlier analysis of P-WbCDPKI and P-hisH1. The enzyme is strongly stimulated by a combination of Mg^{2+} and Ca^{2+}, without being dependent on either of them and is also unaffected by calmodulin and fluphenazine. Orthovanadate strongly inhibits the enzyme while okadaic acid is a poor inhibitor.

Keywords: *Psophocarpus tetragonolobus*; Leguminosae; Winged bean; Protein phosphatase; Protein dephosphorylation; Enzyme purification

1. Introduction

The phosphorylation status of structural and regulatory proteins plays a key role in the regulation of many eukaryotic cellular processes (Krebs, 1986; Ranjeva & Boudet, 1987). Thus, reversible protein phosphorylation which is sensitively controlled by the mediation of protein kinases (PKs) and protein phosphatases (PPs), is now widely accepted as a primary mechanism of a variety of stimulus-response processes and Hunter has very aptly branded the phosphorylation status of proteins as "a major currency of signal transduction pathways" (Cohen, 1992; Hunter, 1995). In general, PPs are well conserved proteins and on the basis of their substrate specificity, metal ion requirements and inhibition profile (sensitivity to heat stable protein inhibitors (I-1 and I-2), okadaic acid or microcystin LR), they have been grouped into four major classes, i.e. PP1, PP2A, PP2B and PP2C (Cohen, 1989). In plants, PPs have not received much attention until the recent realization that these enzymes, like PKs, play crucial roles in regulating a vast array of cellular processes and the presence of all the four

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major classes of PPs, has also been documented. In addition, the application of molecular cloning techniques has led to the identification of an increasing number of non-classical phosphatases with novel structural features which deviate from the major classes of PPs, as indicated by their primary structure deduced from the corresponding gene sequence (MacKintosh & Cohen, 1989; MacKintosh, Coggins & Cohen, 1991; Luan, Li, Rusnak, Assmann & Schreiber, 1993; Wera & Hemmings, 1995).

Our recent isolation and characterization of two calmodulin-like domain PKs, i.e. WbCDPK (Saha & Singh, 1995) and its isoform WbPK (Ganguly & Singh, 1998) (designated here as WbCDPKI and WbCDPKII, respectively), from the same soluble fraction of winged bean (*Psophocarpus tetragonolobus*) shoot extract, have given an impetus to the studies of their regulation and physiological function(s). Earlier studies have shown that WbCDPKI is down-regulated by autophosphorylation and we have pointed out the physiological relevance of a protein phosphatase in the same tissue for the reactivation of the phosphorylated enzyme (Saha & Singh, 1995). This prompted us to undertake a systematic study of winged bean protein phosphatases as nothing is known about the PPs of this plant. Here we describe the identification of a protein phosphatase (WbPP) in the soluble fraction of winged bean shoot extract and its purification. Preliminary characterization shows that autophosphorylated WbCDPKI (P-WbCDPKI) and phosphohistone H1 (P-hisH1), prepared by using the homologous WbCDPKII, are good substrates of the purified enzyme.

Table 1
Orthovanadate sensitive phosphatase activity in the soluble extract

Substrate	Orthovanadate added (mM)	Activity (pmol·min^{-1}·mg^{-1})
PhisH1*	none	5.25 ± 0.1
PhisH1*	0.1	0.37 ± 0.007
P-WbCDPKI†	none	3.9 ± 0.08
P-WbCDPKI†	0.1	0.22 ± 0.01

The soluble extract (100 000*g* supernate) were assayed for phosphatase, using 0.5 μg of [^{32}P]hisH1 and [^{32}P]-WbCDPKI as substrates. A total reaction vol. of 50 μl contained 20 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg·ml^{-1} BSA, a cocktail of protease inhibitors [leupeptin (1 μg·ml^{-1}), aprotinin (0.2 units·ml^{-1}), pepstatin A (2 μg·ml^{-1}), 0.4 mM benzamidine and 0.4 mM PMSF] in 50 mM Tris·HCl buffer, pH 7.5. Reactions, in triplicates, were started by the addition of 10 μl of the extract (100×diluted; 0.05 mg·ml^{-1}) and incubated at 25° for 1 min and stopped with 10 mM silicotungstic acid. Orthovanadate was added, as indicated. Activity is the mean ± S.D. of three independent samples.*Histone H1 phosphorylated by WbCDPKII.†Autophosphorylated WbCDPKI.

2. Results and discussion

2.1. Identification of a winged bean protein phosphatase which catalyses the dephosphorylation of autophosphorylated WbCDPKI

Using P-WbCDPKI as the test substrate, a systematic search has been made for a protein phosphatase which catalyses the dephosphorylation of the autophosphorylated WbCDPKI and such an enzyme has been identified in the soluble fraction of winged bean shoot. As presented in Table 1, P-hisH1 is as good a substrate of the winged bean phosphatase (WbPP) as P-WbCDPKI and both of the reactions are strongly inhibited by 0.1 mM orthovanadate. Since classical protein phosphoserine phosphatases are known to be insensitive to orthovanadate inhibition (Wera & Hemmings, 1995), WbPP may not belong to any of the four major families of PPs, i.e. PP1, PP2A, PP2B and PP2C, the presence of which have also been established by us (Ganguly and Singh, unpublished data), using phosphorylated phosphorylase kinase as the substrate and various inhibitors, such as the heat stable protein inhibitors, I-1 and I-2 and okadaic acid, as outlined by Cohen and his group (Cohen, 1989; MacKintosh & Cohen, 1989; MacKintosh et al., 1991).

2.2. Purification of WbPP

2.2.1. Preparation of 100 000g supernate (soluble fraction)

The soluble fraction (100 000*g* supernate) of the tissue extract has been prepared from 50 g of winged bean shoot in buffer A [50 mM Tris·HCl (pH 8.0) and 2 mM 2-mercaptoethanol], as described in Section 3. It has 752 enzyme units with a specific activity of 4.7 units/mg.

2.2.2. DEAE-cellulose column chromatography

The soluble extract, containing 752 enzyme units and 160 mg protein, was loaded on a DEAE-cellulose column (2×13 cm), equilibrated with buffer A. After thorough washing of the column to remove the unbound proteins, a linear NaCl gradient (0 to 0.5 M) was applied and the phosphatase was eluted in the descending portion of the second broad protein peak approximately between 0.15 and 0.3 M NaCl concentrations [Fig. 1(a)]. The active fractions (18–25) were pooled and concentrated.

2.2.3. Gel filtration chromatography

The pooled sample, containing 42 mg of protein with 440 enzyme units and a specific activity of 10.5 units/mg, was loaded on a Sephacryl S-200 col-

umn (1.5×85 cm), equilibrated with the same buffer. The enzyme appeared in the first major protein peak [Fig. 1(b)] and the fractions (19 to 23), having high phosphatase activity were pooled and supplemented with $MgCl_2$ to a final concentration of 10 mM. It contained 4.8 mg of protein with a total phosphatase activity of 195 units and a specific activity of 41 units/mg.

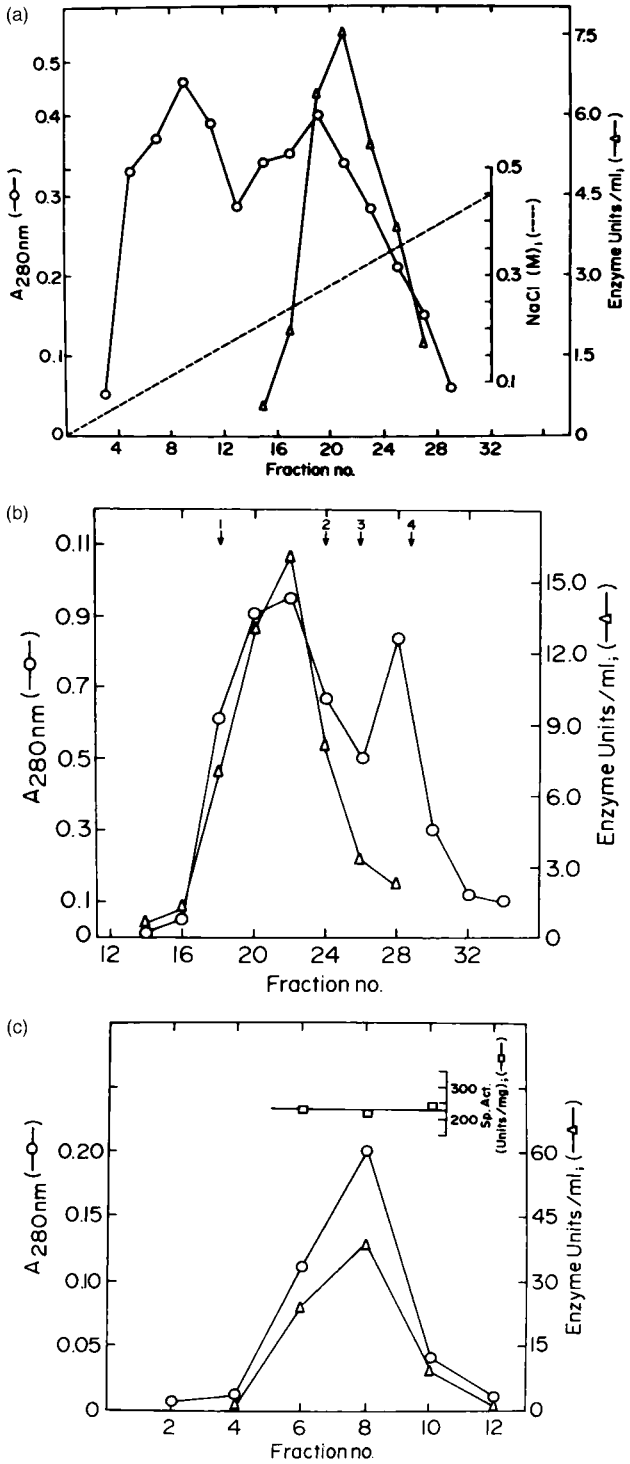

Fig. 1. Purification of WbPP. (a) DEAE-cellulose chromatography. The phosphatase was eluted from the DEAE-cellulose column by a linear gradient of NaCl (0 to 0.5 M) and 5 ml fractions were collected. Active fractions, (18 to 25, eluted between 0.15 and 0.3 M NaCl) are pooled. Details are given in Section 2 (– ◦ –, $A_{280\ nm}$; – △ –, enzyme units·ml^{-1}; - - -, NaCl concentration). (b) Sephacryl S-200 column chromatography. In the gel filtration on Sephacryl S-200 column, 2.5 ml fractions are collected and active fractions (19 to 23) are pooled. The arrows indicate the elution positions of standard M_r proteins: 1, aldolase (158 000); 2, BSA (67 000); 3, ovalbumin (45 000); 4, chymotrypsinogen (25 000). Details are given in Section 2 (– ◦ –, $A_{280\ nm}$; – △ –, enzyme units·ml^{-1}). (c) Affinity chromatography on thiophosphohistone-Sepharose. The bound phosphatase was eluted with 0.2 M NaCl and 1 ml fractions collected. Fractions, having almost constant sp. act. (5–10) were pooled. Details are given in Section 2 [– ◦ –, $A_{280\ nm}$; – △ –, enzyme units·ml^{-1}; – □ –, sp. act. of WbPP (units·mg^{-1})].

2.2.4. *Affinity chromatography*

The pooled sample from the previous step was then applied to a thiophosphorylated histone-Sepharose column (5 ml bed volume), equilibrated with buffer A, containing 10 mM $MgCl_2$, the presence of the latter being essential to facilitate binding of the enzyme to the affinity matrix. After washing the column with the same buffer, the enzyme was eluted as a sharp peak by 0.2 M NaCl [Fig. 1(c)]; the peak fractions (5 to 10) were pooled and concentrated by Centriprep-10 (Amicon). Fig. 1(c) also shows that in this step, the specific activity of the enzyme (*ca.* 220 units/mg) across the peak is more or less constant, indicating that the preparation is essentially pure.

Table 2 summarizes a typical purification of the enzyme, starting with 50 g of the tissue. Throughout the course of the purification, instead of P-WbCDPKI, P-hisH1, prepared by using WbCDPKII, has been used as the substrate for monitoring the activity of WbPP. By adopting this procedure, 300 μg of purified enzyme was obtained with an overall yield of *ca.* 8% and a low purification factor of *ca.* 44-fold. In our attempt to minimize contaminating kinases, we have collected active phosphatase fractions judiciously, sacrificing a large portions of its activity and thus resulting in major losses (Table 2). Since accurate measurements of enzyme activity in the crude extracts are usually difficult due to the presence of various interfering substances, we have avoided any serious conclusions, based on the low yield as well as the apparently low enrichment factor. Even after its purification to apparent homogeneity, the WbPP preparation is still unstable as evident from its gradual loss of activity on storage at 4°C (data not shown).

Table 2
Summary of the purification of WbPP

Steps	Total protein (mg)	Total activity (units)	Sp. activity (units·mg$^{\pm 1}$)	Recovery (%)
Shoot extract (100 000*g* supernate)	160	752	4.7	100
DEAE-cellulose	42	440	10.5	58
Sephacryl S-200	4.8	195	41	26
Thiophosphohistone-Sepharose	0.3	62	206	8

A procedure for the purification of WbPP, starting with 50 g of winged bean shoot, has been standardized. Phosphatase assays are done in triplicate with [^{32}P]-hisH1 as the substrate, in a reaction mixture containing 50 mM Tris·HCl (pH 7.5), BSA (1 mg·ml^{-1}), 20 mM $MgCl_2$ and 1 mM $CaCl_2$ as described in Section 3.

2.3. Homogeneity and size of the purified WbPP

Single protein bands are observed when the purified WbPP preparation is analysed by alkaline PAGE in the presence and absence of SDS, followed by Coomassie blue staining [Fig. 2(a) and (b)] and activity staining using BCIP and NBT reveal that the Coomassie blue-stained protein band [Fig. 2(b)] is a phosphatase [Fig. 2(c)]. These findings, taken together with the earlier results of constant specific activity of the peak fractions, eluted from the thiophosphorylated histone-Sepharose affinity column [Fig. 1(c)], attest to the essential homogeneity of the preparation.

From the results of SDS-PAGE analysis [Fig. 2(a)], an approximate M_r of 92 000 has been estimated for the purified WbPP while a value of *ca.* 85 000 is obtained for the native enzyme, by gel permeation chromatography studies under non-denaturating conditions (data not shown). This clearly shows that WbPP is a single polypeptide enzyme.

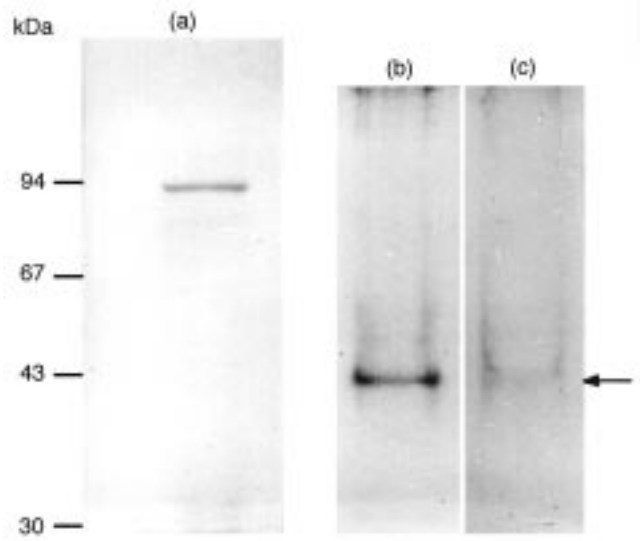

Fig. 2. Homogeneity of the purified WbPP. (a) SDS-PAGE. The purified WbPP (3 μg) was subjected to SDS-PAGE (9% acrylamide) under reducing conditions and stained by Coomassie Blue R-250. The numbers on the left margin indicate the M_r of the standard marker proteins. (b) Alkaline PAGE (under non-denaturating conditions). WbPP (6 μg) is analysed by PAGE (7% acrylamide) under non-denaturating conditions and the gel stained by Coomassie Blue R-250. (c) Activity staining on polyacrylamide gel (7% acrylamide). Purified WbPP (20 μg) was subjected to alkaline PAGE (under non-denaturating condition) at 4° and the phosphatase activity was stained by BCIP/NBT, as described in Section 3. The arrow (←) indicates the position of WbPP in (b) and (c), the electrophoretic runs being carried out under identical conditions.

2.4. Bivalent metal ion requirement of WbPP

Table 3 represents the effect of bivalent metal ions on the activity of WbPP. Supplementation of the reaction mixture with 1 mM $CaCl_2$ and 20 mM $MgCl_2$ results in *ca.* 3.6-fold increase in the phosphatase activity. However, a combination of EGTA and EDTA lowers the activity only nominally, indicating that WbPP is not Ca^{2+} and Mg^{2+} dependent, but is stimulated by these metal ions. In the presence of the chelating agents, the enhancement of the activity by combination of Ca^{2+} and Mg^{2+} is about 2.6 fold, instead of the higher value of about 3.6 fold observed in the absence of the chelators. Individually, Ca^{2+} and Mg^{2+} shows low stimulation. The effect of calmodulin (CaM) has also been tested in order to distinguish WbPP from the classical eukaryotic PP2B type of phosphatase which is characteristically stimulated by CaM. Table 3 shows that at 1×10^{-4} mM concentration, CaM has no effect on the WbPP activity but it is slightly inhibited by 1×10^{-2} mM CaM and the inhibition may be attributed to the lowering of free Ca^{2+} in the reaction mixture due to its binding to CaM.

The concentration of Ca^{2+} required for the stimulation of the phosphatase activity appears to be much higher than the physiological levels (by several hundred folds) and we could not demonstrate any significant stimulation of PP activity at lower concentrations of Ca^{2+} (data not shown).

Table 3
Effect of bivalent metal ions and CaM on WbPP

Additions (mM)	Sp. act. (units·mg^{-1})	Relative act. (%)
None	56	100
$MgCl_2$ (20) + $CaCl_2$ (1)	203	363
EDTA (0.5) + EGTA (0.5)	52	92
EDTA (0.5) + EGTA (0.5) + $MgCl_2$ (10)	74	133
EDTA (0.5) + EGTA (0.5) + $MgCl_2$ (20)	83	148
EDTA (0.5) + EGTA (0.5) + $CaCl_2$ (1)	73	131
EDTA (0.5) + EGTA (0.5) + $CaCl_2$ (5)	77	137
EDTA (0.5) + EGTA (0.5) + $MgCl_2$ (20) + $CaCl_2$ (1)	148	265
EDTA (0.5) + EGTA (0.5) + $MgCl_2$ (20) + $CaCl_2$ (1) + CaM (10^{-4})	146	261
EDTA (0.5) + EGTA (0.5) + $MgCl_2$ (20) + $CaCl_2$ (1) + CaM (10^{-2})	116	207

WbPP is assayed in a reaction mixture as described in Table 2, without any exogenous divalent metal ions, using [^{32}P]-hisH1 as the substrate. The concentrations (mM) of metal ions, EDTA, EGTA and calmodulin (CaM), wherever added, are indicated in parenthesis. Sp. act. are calculated from the mean values of triplicate assays.

2.5. Effect of phosphatase inhibitors on WbPP

WbPP is almost completely inhibited by 100 μM sodium orthovanadate with only slight inhibitions at lower concentrations down to 20 μM (Table 4), but 5 mM NaF has very low inhibitory effect. On the other hand, okadaic acid shows about 25 and 50% inhibition at 5 and 12.5 μM concentrations, respectively. Table 4 also shows that fluphenazine, a CaM antagonist has no effect on the activity of the phosphatase at 50 μM concentration.

The inhibitor profile of WbPP does not conform with any of the known major protein phosphoserine phosphatases as all these major PPs are insensitive to orthovanadate inhibition and the degree of WbPP's sensitivity to okadaic acid is also different from those of the other PPs as both PP1 and PP2A are highly sensitive to this inhibitor and the IC_{50} for PP2B, which is about 5 μM, is much lower than that of WbPP (12.5 μM) while PP2C is not inhibited by okadaic acid (Cohen, 1989).

2.6. Substrate specificity of WbPP

Among the various compounds tested as substrates of the purified WbPP, P-WbCDPKI and P-hisH1, prepared by WbCDPKII are found to be good exogenous substrates while phosphohistone H1 and phosphocasein, prepared by using cAMP-dependent protein kinase, are very poor and totally inactive, respectively (Table 5).

2.7. Identification of P_i released by WbPP from the substrates

When [^{32}P]-WbCDPKI is incubated with WbPP and the reaction mixture is analysed by TLC on PEI-cellulose, followed by autoradiography, the release of $^{32}P_i$ is observed (Fig. 3, lane 2). The control without WbPP treatment, shows the background with negligible release of $^{32}P_i$ (lane 1). In the case of [^{32}P]-hisH1 also, similar results have been obtained (data not shown). Since both P-WbCDPKI and P-hisH1 have previously been shown to have only phosphoserine as the phos-

Table 4
Effect of some common phosphatase inhibitors on purified WbPP

Inhibitors	Concentrations (μM)	Sp. act. (units·mg^{-1})	Relative act. (%)
None	–	126	100
Sodium orthovanadate	20	111	88
Sodium orthovanadate	100	2	1.6
Okadaic acid	5	95	75
Okadaic acid	12.5	65	52
Fluphenazine	50	125	99
Sodium fluoride	5×10^3	108	86

WbPP is assayed using [^{32}P]-hisH1 as substrate, in a reaction mixture containing 50 mM Tris·HCl (pH 7.5), BSA (1 mg·ml^{-1}), 20 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM EDTA and 0.5 mM EGTA in a total vol. of 50 μl, as described in Table 2. Inhibitors are added in concentrations, as indicated. Sp. act. are calculated from the mean value of triplicate assays.

Table 5
Substrate specificity of purified WbPP

Substrate	Sp. act. (units·mg^{-1})	Relative act. (%)
P-hisH1*	126	100
Phosphohistone H1†	10.7	8
P-WbCDPK (Autophosphorylated)	80.5	64
Phosphocasein†	Not detectable	–

Various [^{32}P]-labeled phosphoproteins (0.5 μg each), have been tested as substrates of WbPP and the enzyme is assayed, as described in Section 3. Sp. Act. are calculated from the mean value of triplicate assays.*Phosphorylated by WbCDPKII.†Phosphorylated by PKA.

phorylated amino acid residue (Saha & Singh, 1995; Ganguly & Singh, 1998), the phosphoserine has to be the source of P_i. Therefore, WbPP is adjudged to be a protein phosphoserine phosphatase.

In the present studies, we have identified a soluble protein phosphatase from winged bean shoot and after its purification, an attempt has been made to classify it on the basis of its biochemical parameters: (a) its weak inhibition by okadaic acid but high sensitivity to orthovanadate; (b) its stimulation by a combination of Ca^{2+} and Mg^{2+}, without being truly dependent on either of them; (c) its insensitivity to CaM and fluphenazine; (d) its substrate range and (e) the monomeric nature of the enzyme. As pointed out earlier, it is obvious that WbPP does not fit into the existing system of PP classification; because typical PP1 and PP2A are strongly inhibited by okadaic acid and possess distinct subunit structures; PP2B is Ca^{2+} dependent, stimulated by CaM and inhibited by CaM antagonists and PP2C is Mg^{2+} dependent and insensitive to okadaic acid (Cohen, 1989). However, relaxing these criteria of classification to some extent, one can consider WbPP as an intermediate between PP2B and PP2C, taking its weak inhibition by okadaic acid and stimulation by Ca^{2+} as partial characteristics of PP2B and the stimulation by Mg^{2+} and lack of quaternary structure as partial characteristics of PP2C. Such deviations from classical phosphatases have been reported for a number of atypical PPs, identified recently by molecular cloning (Wera & Hemmings, 1995). These include the Ca^{2+} binding PP2C-homologue which is implicated in abscisic acid responses in *Arabidopsis* (Leung, Bouvier-Durand, Morris, Guerrier, Chefdor & Giraudat, 1994; Meyer, Leube & Grill, 1994; Armstrong, Leung, Grabov, Brearley, Giraudat & Blatt, 1995), the phosphatase from *Arabidopsis* containing PP2C catalytic domain along with a kinase interacting domain (Stone, Collinge, Smith, Horn & Walker, 1994) and the Mg^{2+}-dependent Ca^{2+}-stimulated bovine mitochondrial phosphatase (Lawson, Niu, Browning, Trong, Yan & Reed, 1993). In general, discovery of such enzymes with hybrid characteristics will be a matter of common experience as an outcome of the ever increasing application of the powerful recombinant DNA techniques in all aspects of modern biology.

Fig. 3. Detection of $^{32}P_i$ released by WbPP from the [^{32}P]-labeled phosphoprotein. [^{32}P]-WbCDPKI was incubated with WbPP in a standard reaction mixture for 20 min and 1 μl of the reaction mixture was analysed by TLC on PEI-cellulose, as described in Section 3. Lane 1: control (no WbPP); lane 2: dephosphorylation of [^{32}P]-WbCDPK by WbPP; the top arrow indicates the position of standard $^{32}P_i$ and origin is indicated by the lower arrow.

As pointed out earlier, the unusual phenomenon of down-regulation of WbCDPKI by autophosphorylation (Saha & Singh, 1995), has led us to speculate on the existence of a regulatory feedback circuit, involving a phosphatase for the regeneration of the phosphorylated WbCDPKI. The present finding of WbPP in the same soluble fraction of the winged bean shoot, lends credence to the proposal. Similar interplay between CDPKs and a PP2C-like phosphatase in stress signal transduction has recently been reported in *Arabidopsis* (Sheen, 1996). Thus, WbPP, the first PP from winged bean, may serve as an im-

portant component of a regulatory cascade in the rapidly growing plant tissue.

3. Experimental

3.1. Chemicals

[γ-^{32}P]ATP (>5000 Ci·mmol^{-1}) was obtained from Amersham, U.K. Bovine brain calmodulin (CaM), okadaic acid, histone H1, dephosphorylated casein, adenosine 5′-*O*-(3-thiotriphosphate) [ATP-γ-S], fluphenazine, EDTA, EGTA, pepstatin, leupeptin, aprotinin, PMSF, benzamidine–HCl, Na orthovanadate, PVP (cross-linked) and cAMP-dependent protein kinase were obtained from Sigma. Sepharose CL-4B and Sephacryl S-200 were purchased from Pharmacia and DEAE-cellulose was from Pierce. Other chemicals used were of analytical grade.

3.2. Plant tissue

Winged bean [*Psophocarpus tetragonolobus* (L.) DC.] seeds collected from the plants grown in the Institute garden were germinated at 25°. 5- to 6-day-old shoots were used as starting material.

3.3. Preparation of the winged bean shoot homogenate

Fresh or frozen winged bean shoots were homogenized in 3 vol. of ice cold buffer A [50 mM Tris·HCl (pH 8.0) and 2 mM 2-mercaptoethanol], containing 1.5 mM EDTA, 1 mM PMSF, 6 mM benzamidine, leupeptin (5 μg·ml^{-1}), aprotinin (0.2 units·ml^{-1}), pepstatin A (2 μg·ml^{-1}) and cross-linked PVP (0.1 g·g^{-1} tissue) in a Sorvall Omnimixer, as described in Ganguly and Singh (1998), and the homogenate was passed through several layers of cheese cloth. The filtrate was centrifuged at 9000*g* for 10 min and the supernatant fluid subjected to ultracentrifugation at 100 000*g* for 1 h. The resultant clear supernate was used as the starting extract for the purification of the phosphatase and for which a combination of chromatography steps was standardized, all operations being carried out at 4°.

3.4. Preparation of the protein kinases of winged bean shoot

Preparation of WbCDPKI from winged bean shoot has been described earlier (Saha & Singh, 1995) and WbCDPKII has also been purified from the soluble fraction of the same homogenate, using DEAE-cellulose, hydroxylapatite and Cibacron Blue-Sepharose column chromatography steps, according to Ganguly and Singh (1998).

3.5. Preparation of [^{32}P]-labeled substrates

Purified WbCDPKI was autophosphorylated by using [γ-^{32}P]ATP as described in Saha and Singh (1995), yielding [^{32}P]-WbCDPKI with a sp. act. of 1×10^5 cpm·μg^{-1}. Purified WbCDPKII was used to phosphorylate histone H1 in a reaction mixture of 250 μl total vol., containing 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mg·ml^{-1} BSA, 0.05 mM [γ-^{32}P]ATP (sp. act. 2000 cpm·pmol^{-1}) and histone H1 (0.16 mg·ml^{-1}). The reaction was started by the addition of 2 μg of enzyme (WbCDPKII) and incubated at 25° for 1 h. After stopping the reaction by the addition of 250 μl 50% cold TCA (v/v), the mixture was centrifuged at 10 000*g* for 5 min. The ppt. was washed with acetone (Kato & Bishop, 1972) and finally dissolved in H_2O and stored at −20°. The resultant ^{32}P-hisH1 (sp. act. 5×10^4 cpm·μg^{-1}) was used as substrate. [^{32}P]-histone H1 (sp. act. of 2.5×10^4 cpm·μg^{-1}) and phosphocasein (sp. act. of 2×10^4 cpm·μg^{-1}), were prepared by using cAMP-dependent protein kinase (Kato & Bishop, 1972; MacGowan & Cohen, 1988), instead of WbCDPKII.

3.6. Protein phosphatase assay

The protein phosphatase was routinely assayed using [^{32}P]-hisH1 or [^{32}P]-WbCDPKI as the substrate, in a reaction mixture of 50 μl total vol., containing 50 mM Tris·HCl (pH 7.5), a cocktail of protease inhibitors [leupeptin (1 μg·ml^{-1}), aprotinin (0.2 units·ml^{-1}), pepstatin A (2 μg·ml^{-1}), 0.4 mM benzamidine and 0.4 mM PMSF], BSA (1 mg·ml^{-1}), 20 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.5 mg·ml^{-1} [^{32}P]-labeled substrate. The reaction was started by the addition of the enzyme, incubated for a fixed time (within the linear range) at 25° and stopped by the addition of 50 μl of 20 mM silicotungstic acid in 0.4 M H_2SO_4. It was kept in ice for 15 min and then centrifuged at 13 000*g* for 5 min; 90 μl of the supernatant was counted in a liquid scintillation counter. The phosphatase activity was calculated from the mean value of the triplicate assays.

3.7. Phosphatase unit

One protein phosphatase unit was defined as that amount of enzyme which catalyses the release of 1 pmol of orthophosphate per min from the phosphorylated substrates, under the conditions of assay.

3.8. Preparation of thiophosphorylated histone-Sepharose

Thiophosphorylated histone H1 was immobilized to Sepharose according to Pato and Adelstein (1980), with some modifications as follows: histone H1 was coupled to CNBr-activated Sepharose CL-4B according to Wilchek, Miron and Kohn (1984) and bound histone H1 was thiophosphorylated using WbPK in a reaction mixture, containing the histone-Sepharose slurry, 0.05 M Tris·HCl (pH 7.5), 0.05 mM ATP-γ-S, 0.1 mM orthovanadate, 2.5 mM DTT, 10 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The reaction was carried out overnight by a gentle agitation of the slurry at *ca.* 10° and then the thiophosphorylated histone-Sepharose was washed with 10 vol. of 0.05 M Tris·HCl (pH 7.5) containing 0.5 M NaCl.

3.9. Purification of WbPP from winged bean shoot

For the purification of WbPP, the 100 000*g* supernate of the tissue extract was fractionated on a DEAE-cellulose column, using a linear NaCl gradient (0 to 0.5 M). In the next step, the sample was subjected to gel filtration on Sephacryl S-200, followed by affinity chromatography on a thiophosphorylated histone-Sepharose.

3.10. Activity staining of WbPP

The purified WbPP (20 μg) was subjected to alkaline PAGE under non-denaturating conditions according to Laemmli (1970) at 4°. The gel was washed thoroughly with 50 mM Tris·HCl (pH 8.0), containing 20 mM $MgCl_2$ for *ca.* 1 h with 4× changes. For color development, the procedure of Blake, Johnston, Russell-Jones and Gotschlich (1984), was modified. After thorough washing, the gel was incubated overnight in the dark with a reaction mixture containing 5 mg·ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 10 mg·ml^{-1} Nitroblue tetrazolium (NBT), 20 mM $MgCl_2$ and 1 mM $CaCl_2$ in 0.1 mM Tris·HCl (pH 8.0).

3.11. Detection of P_i released by dephosphorylation

The ^{32}P-labeled protein substrate was incubated with WbPP in a standard reaction mixture as described above. After 20 min, *ca.* 1 μl of the mixture is spotted on polyethyleneimine (PEI)-cellulose plate (Sigma), dried and the released $^{32}P_i$ is separated by TLC in 250 mM K–Pi buffer system at pH 7.6 and $^{32}P_i$ was detected by autoradiography. Control incubations without WbPP and standard $H_3{}^{32}PO_4$ were also analysed by TLC simultaneously.

3.12. Other methods

The M_r of the native enzyme was determined by analytical gel filtration (Laurent & Killander, 1964). Protein was determined by the Coomassie Blue dye binding method of Bradford (1976), with BSA as the standard. SDS-PAGE was carried out under reducing conditions, as described in Laemmli (1970).

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