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CHARACTERIZATION OF A SECOND CALCIUM-DEPENDENT PROTEIN KINASE FROM WINGED BEAN

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Key Word Index—*Psophocarpus tetragonolobus*; Leguminosae; winged bean; signal transduction; protein phosphorylation; calmodulin-like domain protein kinase; Ca^{2+}-dependent protein kinase; calmodulin antagonist.

Abstract—In plants, Ca^{2+} has emerged as the predominant second messenger for signal transduction, as cyclic nucleotides are not known to play any significant role in this system. Earlier, we characterized an interesting Ca^{2+}-dependent protein kinase, WbCDPK (winged bean calmodulin-like domain protein kinase), from the soluble fraction of winged bean (*Psophocarpus tetragonolobus*) shoot extract. Here an isoform of WbCDPK is purified to apparent homogeneity from the same winged bean shoot extract. It is a single polypeptide chain protein–serine kinase, having an M_r of about 70,000 and like WbCDPK, its preferred substrates are histone H1, syntide 2 and MLC-peptide (a synthetic myosin light chain related peptide) and it is totally dependent on Ca^{2+} for its activity, but exogenous calmodulin (CaM) does not stimulate it. However, it is strongly inhibited by CaM antagonists, indicating the presence of a CaM-like domain, as in WbCDPK. The two enzymes do not cross react immunologically and the isoform differs significantly from WbCDPK in its apparent inability to catalyse the autophosphorylation reaction, which is known to cause down-regulation of substrate phosphorylation in the case of WbCDPK.

INTRODUCTION

Many physiological processes such as cellular metabolism, cell division and differentiation are regulated by reversible phosphorylation of several cellular proteins in response to a diverse array of external stimuli [1, 2]. In contrast to the highly sophisticated signal transduction systems of animals, a proper understanding of this phenomenon in plants has developed only in the recent past. Nevertheless, with the isolation and characterization of an increasing number of protein kinases from higher plants, rapid progress is also being made towards deciphering the signal transduction pathways in this system. Ca^{2-} is now widely accepted to have a key role as one of the second messengers in stimulus–response processes in plants and several studies indicate that it exerts its influence by activating Ca^{2+}-dependent protein kinases [3, 4]. Among these, CDPK (calmodulin-like domain protein kinase/Ca^{2+}-dependent protein kinase) is a novel class of protein kinase. It has been characterized in several higher plants and lower eukaryotes, such as soybean [5], apple [6], oat [7], ground nut [8], *Arabidopsis* [9], maize [10, 11], winged bean [12]. CDPKs are widely distributed in plants and several isoenzymes have also been identified. In *Arabidopsis* alone, about 12 such isoforms, with M_r in the range of 55,000–72,000, have been identified [13].

Unlike other Ca^{2+}-dependent protein kinases, like Ca^{2+}/calmodulin-dependent protein kinase or the protein kinase C, the members of the CDPK family require only Ca^{2+} for their activity [14]. In addition to the conserved catalytic domain located in the N-terminal region, CDPK contains a Ca^{2+}-binding CaM-like EF hand motif in the C-terminal part of the polypeptide chain and it probably functions as an efficient sensor of intracellular Ca^{2+} flux. This structural novelty distinguishes it from other Ca^{2+}-dependent protein kinases and this also serves as the diagnostic feature of CDPKs [15]. Because of this, some workers prefer to call it "CaM-like domain protein kinase" instead of "Ca^{2+}-dependent protein kinase". The other interesting aspect of the structural organization of CDPKs is the junction sequence which links up the catalytic domain and the CaM-like domain. This is the putative auto-inhibitory sequence which masks the active site of CDPKs under conditions of normal intracellular Ca^{2+} concentrations. With an increase in intracellular Ca^{2+} level, the Ca^{2+}-ligated

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CaM-like domain binds the auto-inhibitory sequence and the enzyme becomes active. These features make this class of protein kinases quite unique and a central role of CDPKs in Ca^{2+}-mediated signal transduction pathways is an attractive hypothesis.

Earlier work from this laboratory has shown that the winged bean CDPK (WbCDPK) isolated from the soluble fraction of the winged bean shoot extract is unusual in being down-regulated by auto-phosphorylation which is stimulated by its substrates, histone H1 (H1) and myosin light chain related peptide (MLC-peptide) [12] and the occurrence of this interesting CDPK in the fast growing winged bean shoot, has further highlighted the importance of Ca^{2+}-mediated signaling in this rapidly growing tissue. During the purification of WbCDPK, we were aware of the presence of at least another Ca^{2+}-dependent protein kinase in the extract and this prompted us to look for the second protein kinase. Here, we report its purification from the same 100,000 *g* supernatant of the winged bean shoot extract and designated it as WbPK (Winged Bean Protein Kinase), to differentiate it from WbCDPK. Characterization of WbPK suggests it to be another CDPK, an isoform of WbCDPK.

RESULTS AND DISCUSSION

Purification of a second Ca^{2+}-dependent protein kinase from winged bean shoot

The soluble fraction of the winged bean shoot extract is fractionated on a DEAE-cellulose column, as described in the Experimental section and the two kinases, WbPK and WbCDPK co-elute between 0.2–0.3 M NaCl (Fig. 1a). But in the next step, i.e. the hydroxylapatite column chromatography, they are separated as two distinct activity peaks (Fig. 1b). The fraction eluted from the hydroxylapatite column at the lower phosphate concentration of 25 mM K-Pi represents WbPK, whereas WbCDPK is recovered by elution by linear gradient of 25–300 mM K-Pi [12], and both of them are further processed separately to yield purified WbPK and WbCDPK from the same tissue extract. The WbPK fraction from the hydroxylapatite column is loaded on a Cibacron Blue–Sepharose column and it binds to the column in a Mg^{2+}-dependent manner, from where it is eluted by 7 mM EDTA (Fig. 1c). The resultant WbPK preparation is essentially free from WbCDPK since the latter does not bind to Blue–Sepharose under the present experimental conditions. However, only the fractions with high specific activity are taken, sacrificing other fractions with low specific activity, resulting in a significant increase in the specific activity, but with overall poor yield.

As summarized in Table 1, 120 μg of the purified protein kinase is obtained from 60 g of tissue, with an overall recovery of *ca* 5% of protein kinase present in the soluble fraction of the tissue extract. The purification has been monitored using syntide 2 as the substrate.

Homogeneity and size of purified WbPK

A single protein band is obtained from the purified enzyme by SDS–PAGE, and Coomassie blue staining (Fig. 2a), indicating apparent homogeneity of the preparation. The M_r of WbPK is estimated to be *ca* 70,000 by SDS–PAGE and 68,000 by gel filtration (Fig. 2b) as well as by sucrose density gradient centrifugation (data not shown), attesting to the monomeric nature of the enzyme. The single protein band with M_r of 70,000 by SDS–PAGE of fraction No. 50, i.e. the peak fraction of the eluate from the gel filtration column (Inset, Fig. 2b), is also consistent with this conclusion.

Therefore, WbPK is obviously larger than WbCDPK (M_r 60,000) and also it does not seem to be as highly susceptible to endogenous protease(s) as WbCDPK, which usually yields a doublet when subjected to SDS–PAGE [12]. The addition of a cocktail of protease inhibitors to the homogenization buffer as well as other precautionary measures taken at the time of tissue homogenization and subsequent steps, are considered to be adequate to prevent interference by proteases during its purification.

Identification of phosphoserine in histone H1, phosphorylated by WbPK

When [^{32}P]-histone H1 is prepared by using purified WbPK and [γ-^{32}P]ATP, subjected to acid hydrolysis and followed by analysis for phosphorylated amino acid residue by thin layer electrophoresis, only [^{32}P]-phosphoserine has been identified (Fig. 3), showing that WbPK is a protein-serine kinase.

Substrate specificity and Ca^{2+} requirement of purified WbPK

Various proteins and synthetic peptides have been tested as possible substrates of WbPK and among them, syntide 2, MLC-peptide and lysine-rich histones (H1 and HIII S) are found to be the preferred exogenous substrates whereas angiotensin, kemptide, histone VIII S, casein, phosvitin, poly glu-tyr and BSA are very poor or inactive (Table 2).

Even in the absence of exogenous Ca^{2+}, the purified WbPK catalyses the phosphorylation of both H1 (Fig. 4a, lane 2) and syntide 2 (Fig. 4b, lane 5), indicating the apparent lack of Ca^{2+} requirement for its activity. However, EGTA drastically inhibits phosphorylation of both H1 (lane 3) and syntide 2 (lane 6) and the inhibition is completely reversed by exogenous Ca^{2+} (lanes 4 and 7), indicating that the enzyme is Ca^{2+}-dependent. No phosphorylated band is detected in lane 1, which is the control without substrate.

Detailed studies as presented in Table 3, reaffirm that the enzyme is highly active even in the absence of

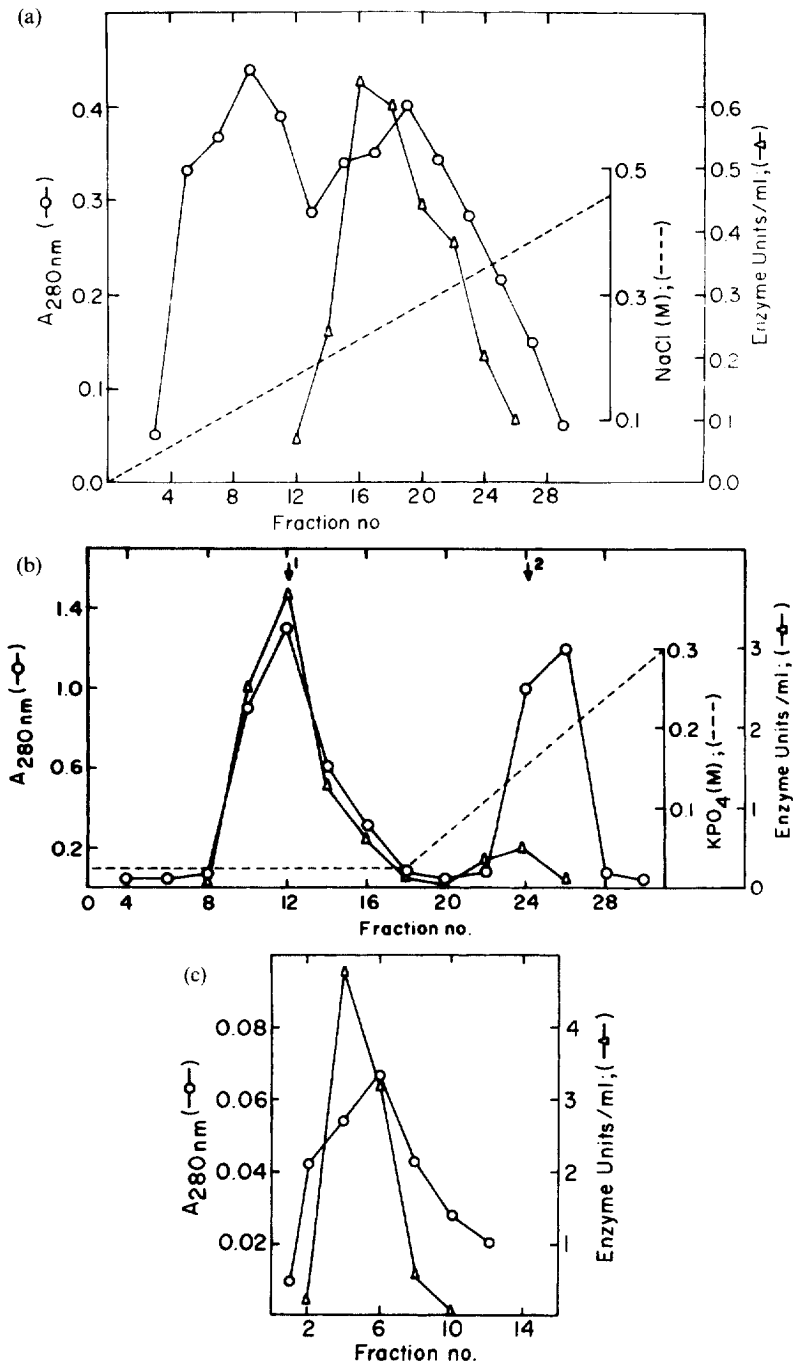

Fig. 1. Purification of WbPK from winged bean shoot. Throughout the purification of WbPK, protein kinase was assayed as described in the Experimental section, using syntide 2 as the substrate. (a) *DEAE-cellulose column chromatography*. The crude extract was loaded on a DEAE-cellulose column and bound proteins were eluted with a linear gradient of 0–0.5 M NaCl. Fractions (14–22), were pooled. (b) *Hydroxylapatite column chromatography*. Pooled fractions from (a) were applied to a hydroxylapatite column and bound proteins were eluted with 0.025 M K-Pi followed by a linear gradient of 0.025-0.3 M K-Pi. Arrows 1 and 2 represent activity peaks of WbPK and WbCDPK, respectively. Fractions 9–15 were pooled for further purification of WbPK. (c) *Affinity chromatography on Blue–Sepharose*. The pooled fractions from (b), were subjected to affinity chromatography on Blue–Sepharose column as described in the Experimental section. Fractions (3–6) were pooled and concentrated using Centriprep-10 (Amicon).

exogenous Ca^{2+}, but addition of 0.2 mM EGTA results in 95% inhibition, which is completely reversed by 0.5 mM $CaCl_2$. These findings suggest that the purified WbPK contains tightly bound Ca^{2+}. As CaM is a classical Ca^{2+}-effector protein, the effect of exogenous CaM, at varying concentrations of 0.1 μM,

Table 1. Summary of purification of WbPK from winged bean shoot. The procedure was standardized by using 60 g of tissue and all the assays were done using syntide 2 as the substrate, in a reaction mixture 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 ATP (Sp. Act. 500–2000 cpm $pmol^{-1}$) and 50 μM syntide 2 in a total vol. of 50 μl, by the P81 binding method, as described in the Experimental section

	Total protein (mg)	Total Activity (unit)	Sp. Activity (unit mg^{-1})	Recovery (%)
100,000 *g* Supernate	165	153	0.9	100
DEAE-cellulose	32	130	4.0	85
Hydroxylapatite	9.8	72.5	7.4	42.7
Blue–Sepharose	0.12	8.2	68.4	4.8

1 μM and 10 μM, has been tested on the phosphorylation of syntide 2, and as shown in the same table, instead of an enhancement, there is slight diminution of substrate phosphorylation, with the highest being 24% in presence of 10 μM CaM, which may be due to the binding of Ca^{2+} by the exogenous CaM and thus lowering the concentration of free Ca^{2+} below the optimal level required for WbPK activation.

Effect of CaM antagonists on WbPK

The ineffectiveness of CaM in spite of the Ca^{2+} dependency of WbPK, has led us to examine the possible presence of a CaM-like domain in the enzyme by its sensitivity towards CaM antagonists. Titration of the enzyme by calmidazolium and fluphenazine, which are potent CaM antagonists, results in extensive inhibition of H1 phosphorylation in a dose-dependent manner, calmidazolium appearing to be a more effective inhibitor (Fig. 5a) than fluphenazine (Fig. 5b). Detailed studies show that the IC_{50} (concentration needed for 50% inhibition) of calmidazolium is *ca* 5 μM and it is *ca* 4-fold less than that of fluphenazine (Fig. 5c).

Effects of phospholipids and cyclic nucleotides on WbPK

In view of its Ca^{2+} dependency, we have also examined the effect of phospholipids on WbPK. Both phosphatidylserine and phosphatidylcholine do not have any influence on the activity of the enzyme either in the presence or the absence of Ca^{2+} (data not shown). Since protein kinase C requires phospholipids in addition to Ca^{2+} for its activity, WbPK is not a member of this family and at the same time it cannot be classified as a protein kinase A or protein kinase G also, as both cAMP and cGMP have no effect on the enzyme (data not shown). These observations, taken together with its dependency on Ca^{2+} without being stimulated by free CaM and the concomitant drastic inhibition by CaM antagonists, clearly favour its classification as a new member of winged bean CDPK family, of which WbCDPK is the first member [12].

Autophosphorylation

Several attempts to demonstrate the autophosphorylation reaction in the case of WbPK, at different ATP concentrations (0.5, 5 and 50 μM) and specific activities (2000–5000 cpm.$pmol^{-1}$) in the reaction mixture, failed even after incubation upto 12 h (data not given). As shown in all the lanes of Fig. 4a, no other phosphorylated band than those of H1 is detected and the absence of a phosphorylated band in the M_r range of 70,000 and 60,000 is a clear indication of: (i) the apparent lack of autophosphorylation reaction in the case of WbPK, both in absence (Fig. 4a, lane 1) or presence of substrate H1 (Fig. 4a, lanes 2–4) and (ii) absence of contaminating WbCDPK, as it undergoes a strong autophosphorylation reaction under the same assay conditions and is stimulated in the presence of H1 [12].

Immunoblot analysis of WbPK

In order to look for any immunological relationship between these two protein kinases belonging to the same family, western blot analysis has been carried out, using rabbit anti-WbCDPK immune sera and protein A-alkaline phosphatase to identify the antigen. WbPK is not recognized by the anti-WbCDPK antibody (Fig. 6, lane 2), and the doublet in lane 1 corresponds to WbCDPK, used as control.

Kinetic properties of purified WbPK

Kinetic analyses of WbPK with syntide 2, MLC-peptide, histone H1 and ATP have been carried out and the kinetic constants, K_m and V_{max} have been calculated from the double reciprocal plots and their replots. The family of converging lines obtained with varying concentration of syntide 2 and ATP, when the concentrations of ATP and syntide 2 are kept constant in the respective cases, have been shown (Fig. 7a and 7b).

As seen in Table 4, both syntide 2 and the MLC-peptide are good peptide substrates and H1 is the best among the proteins tested, even though it is not as good as the two peptides. The table also compares the

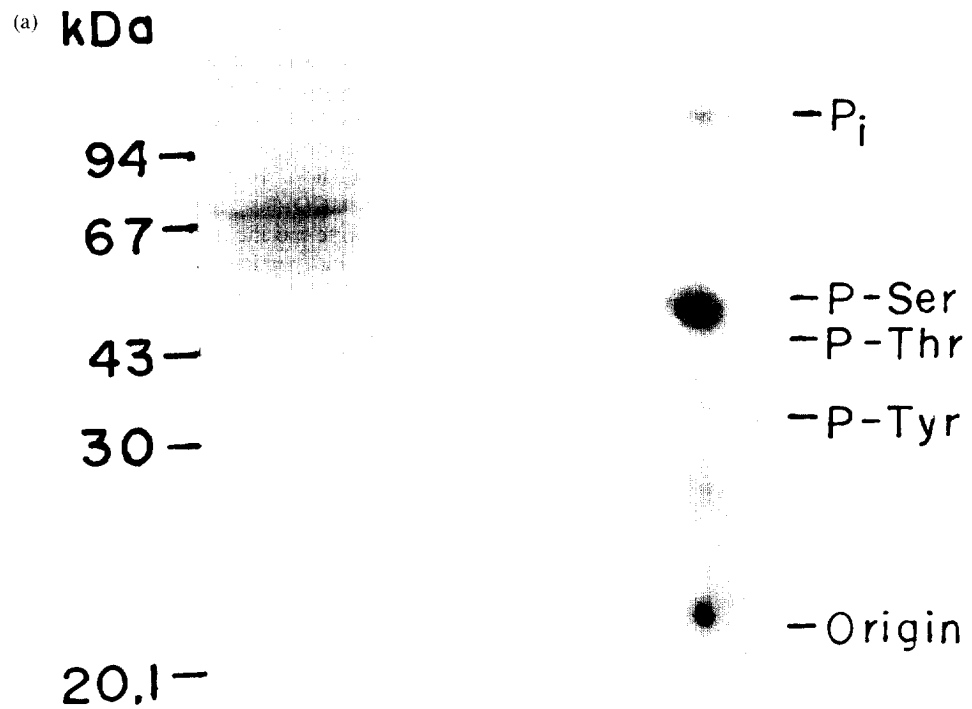

Fig. 3. Identification of phosphoserine. Phosphoamino acid analysis of [^{32}P]-H1, was carried out as described in the Experimental section. Positions of the standard phosphoamino acids, as shown on the right margin, were obtained by ninhydrin staining.

(b)

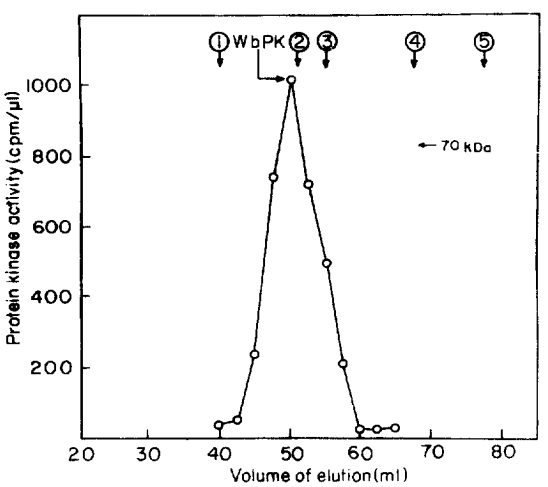

Fig. 2. Homogeneity and size of WbPK. (a) *SDS-PAGE*. The purified WbPK (5 μg protein) was analysed by SDS-PAGE (12% acrylamide). M_rs of the standard proteins are given on the left margin. (b) *Gel filtration*. FPLC gel filtration was carried out in Sephacryl S-100 column (HR 16/60, Pharmacia), in 50 mM Tris.HCl, pH 8.0 buffer containing 100 mM NaCl with a flow rate of 36 ml h^{-1}. Fractions of 1 ml were collected. The elution profile of WbPK is indicated by an arrow. Elution volumes of standard proteins were: (1), Ferritin (M_r 450,000); (2), BSA (M_r 67,000); (3), ovalbumin (M_r 43,000); (4), chymotrypsinogen (M_r 25,000); and (5), cytochrome C (M_r 12,500). (Inset) SDS-PAGE (12 % acrylamide) of the peak fraction, and stained by Coomassie blue R-250. The arrow indicates WbPK (M_r of 70,000).

Table 2. Substrate specificity of WbPK. The protein kinase activity of the purified enzyme was assayed by the P81-binding method as described in Table 1, using various proteins and synthetic peptides as substrate. The concentrations were: Proteins – 0.16 mg ml^{-1}; Synthetic peptides – 50 μM

Substrate	Specific Activity (unit mg^{-1})	Activity (%)
Syntide-2	64.6	100.0
MLC-peptide	57.3	88.0
Histone H1	11.9	18.0
Histone III S	6.8	10.5
Histone VIII S	0.6	0.9
Casein	0.9	1.4
Poly Glu-Tyr	0.3	0.4
Phosvitin	0.0	0.0
Angiotensin	0.5	0.7
Kemptide	0.1	0.1
BSA	0.3	0.4

kinetic constants for WbPK with those of WbCDPK for which values have been taken from our earlier report [12]. The K_m values of 0.035 mg ml^{-1} and 0.07 mg ml^{-1} for H1 in the case of WbPK and WbCDPK respectively, clearly show that H1 is a better substrate for WbPK than for WbCDPK, while syntide 2 and MLC-peptide are better substrates for the latter.

In our studies, the existence of isoenzymes of CDPK

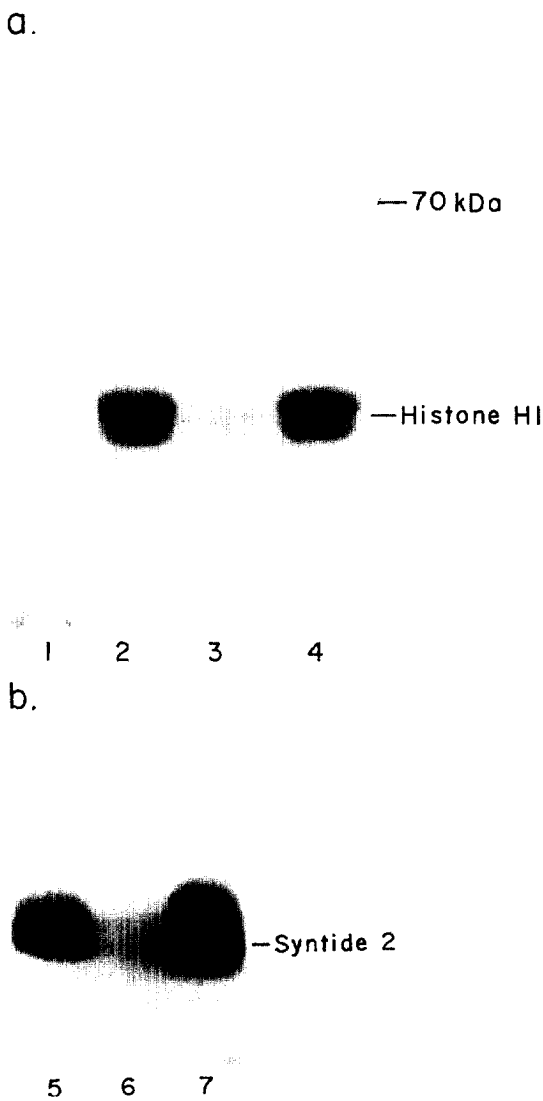

Fig. 4. Effect of Ca^{2+} on substrate phosphorylation. WbPK was assayed using H1 (0.16 mg ml^{-1}) and syntide 2 (50 μM) as substrates, as described in the Experimental section; and if not stated otherwise, the reaction mixture did not contain Ca^{2+} and EGTA. The reaction was terminated after 20 min of incubation by adding SDS- PAGE sample buffer and after SDS–PAGE [12% for (a) and 20% for (b)], the [^{32}P] phosphorylated bands were visualized by autoradiography. (a) Effect of Ca^{2+} on H1 phosphorylation. Lanes: 1, 0.5 mM Ca^{2+} +0.2 mM EGTA, but no exogenous substrate (control); **2**, No Ca^{2+} or EGTA; **3**, 0.2 mM EGTA only; **4**, 0.5 mM Ca^{2+} +0.2 mM EGTA. (**b**) Effect of Ca^{2+} on syntide 2 phosphorylation. **Lanes: 5**, No Ca^{2+} or EGTA; **6**, 0.2 mM EGTA only; **7**, 0.5 mM Ca^{2+} +0.2 mM EGTA.

in winged bean has been firmly established by isolation and extensive characterization, while most of the earlier identifications of such isoforms of CDPKs in higher plants have been based on sequence homology data, obtained by employing recombinant DNA techniques [13, 15, 23]. Earlier, Son *et al.* [24] have reported the characterization of two such isoforms of Ca^{2+}-dependent, phospholipid/CaM-independent protein kinase, designated as "CaPK-1" and "CaPK-2", from the ciliated protozoan *Paramecium tetraurelia*. Similar studies has now been extended to the winged bean also with the characterization of WbPK and the previously purified WbCDPK. In spite of several similarities among these CDPKs, namely, their monomeric nature, substrate specificity, Ca^{2+}-dependence, CaM/phospholipid-independence, the enzymes are clearly distinguishable in terms of size and immunological cross reactivity. Moreover, the apparent lack of autophosphorylation in the case of WbPK highlights its difference from WbCDPK, as the latter is found to be autophosphorylated under the present assay conditions [12]. Hence, WbPK has been considered to be the second member of the winged bean CDPK family and the isolation of two such protein kinases from the same tissue will give an impetus to explore the possible relationship, if any, to the Ca^{2+}-signalling pathway(s) in the rapidly growing winged bean shoot.

EXPERIMENTAL

Plant tissue

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

Chemicals

[γ-^{32}P]ATP (> 5000 Ci $mmol^{-1}$) was obtained from Amersham, U.K. Bovine brain calmodulin (CaM), histones [H1, IIIS, VIII S], casein, phosvitin, fluphenazine, calmidazolium (compound R24571), EDTA, EGTA, DTT, BSA, cAMP, cGMP, pepstatin, leupeptin, aprotinin, PMSF, benzamidine-HCl, polyvinylpolypyrrolidone (cross-linked), phosphatidylserine, phosphatidylcholine, *Staphylococcus aureus* protein A-alkaline phosphate conjugate, 5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium, angiotensin, and the synthetic peptide substrates—syntide 2, kemptide were obtained from Sigma while myosin light chain related peptide (MLC-peptide) was from Peninsula Laboratories, Blue–Sepharose was purchased from Pharmacia, hydroxylapatite from Bio-Rad and DEAE-cellulose from Pierce. Other chemicals used were analytical grade reagents.

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 polyvinylpolypyrrolidone (0.1 g g^{-1} tissue) in a Sorvall Omnimixer. The homo-

Table 3. Effect of Ca^{2+} and CaM on WbPK. The protein kinase was assayed without EGTA and $CaCl_2$ as described in the Experimental section, with syntide 2 as the substrate. EGTA, $CaCl_2$ and calmodulin (CaM) were only added in the reaction mixtures, when required, as given in parenthesis

Effectors (Added)	Concentration (mM)	Sp. Act (units mg^{-1})	Activity (%)
None		36.7	100
EGTA	0.2	2.0	5
EGTA + $CaCl_2$	0.2 + 0.5	42.0	114
EGTA + $CaCl_2$ + CaM	$0.2 + 0.5 + 1 \times 10^{-4}$	35.6	97
EGTA + $CaCl_2$ + CaM	$0.2 + 0.5 + 1 \times 10^{-3}$	35.2	96
EGTA + $CaCl_2$ + CaM	$0.2 + 0.5 + 1 \times 10^{-2}$	27.8	76

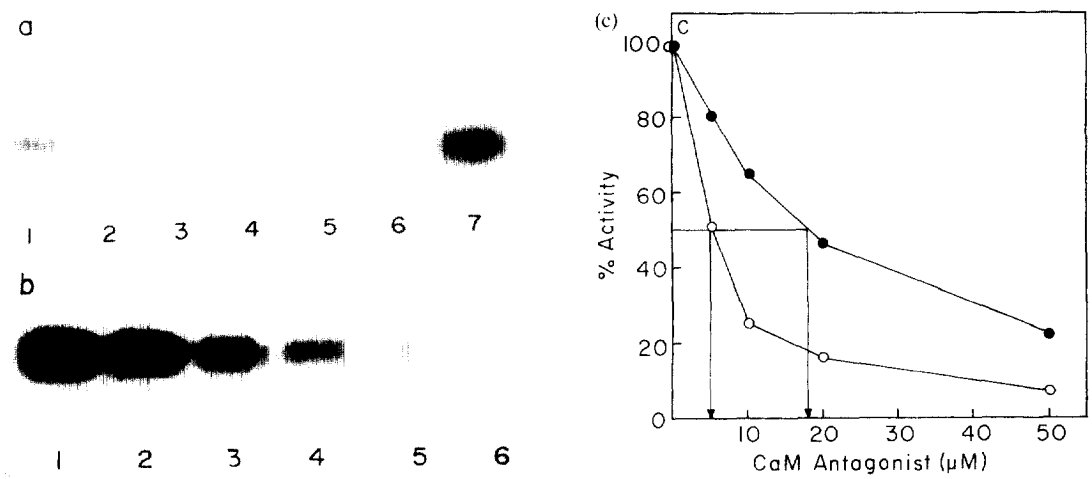

Fig. 5. Inhibition of WbPK activity by CaM antagonists. WbPK was assayed by incubating the reaction mixture for 20 min as described in the Experimental section with H1 as substrate, in the presence or absence of CaM antagonists. [^{32}P]-H1 bands were analysed by autoradiography after SDS–PAGE (12%) and quantitated by gel assay as described in the Experimental section. (a) Inhibition by calmidazolium. **Lanes: 1**, 5 μM calmidazolium; **2**, 10 μM calmidazolium; **3** & **4**, 20 μM calmidazolium; and **5** & **6**, 50 μM calmidazolium; **7**, No addition (control). (b) Inhibition by fluphenazine. **Lanes: 1**, No addition (control); **2**, 5 μM fluphenazine; **3**, 10 μM fluphenazine; **4**, 20 μM fluphenazine; **5** & **6**, 50 μM fluphenazine. (c) Inhibition profile of WbPK activity. A plot of % WbPK activity (activity relative to the control when no antagonist is used) versus antagonist concentration. ○ represents activity in presence of calmidazolium; ● represents activity in presence of fluphenazine; the arrows indicate the concentrations of respective antagonist needed for 50% inhibition of WbPK activity.

genate is passed through several layers of cheese cloth and the filtrate centrifuged at 9000 *g* for 10 min. The supernatant fluid is subjected to ultracentrifugation at 100,000 *g* for 1 h. The resultant clear supernate was designated as crude extract for the purification of the protein kinase employing combinations of chromatography steps, all operations being carried out at 4°.

Purification of WbPK

For the purification of WbPK, the crude extract was loaded on a DEAE-cellulose column (2 × 13 cm), pre-equilibrated in buffer A and after extensive washing with the same buffer the bound proteins were eluted by a linear gradient of NaCl (0–0.5 M), in the same manner as previously described for WbCDPK [12]. The active fractions were pooled and dialysed extensively against buffer A before loading on a hydroxylapatite column (1.5 × 13 cm), equilibrated with the same buffer. The unbound proteins were washed by buffer A and the enzyme eluted by 25 mM K-Pi in the same buffer. The eluted fractions were pooled for further purification of WbPK and a linear gradient of K-Pi (25–300 mM) was used for the recovery of WbCDPK [12]. The pooled fraction, containing WbPK was dialysed against buffer A, containing 2 mM $MgCl_2$ and loaded on a Cibacron Blue–Sepharose column (2 ml, bed vol.), pre-equilibrated with the same buffer. After extensive washing, WbPK was eluted with 7 mM EDTA in buffer A and the fractions with high specific activity were pooled.

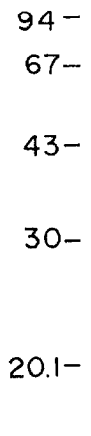

Fig. 6. Immunoblot analysis. The immunoblot analysis was conducted with rabbit antiserum against WbCDPK. Protein A-alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium were used, as described in the Experimental section, for the visualization of the protein bands. **Lanes:** *1*, purified *WbCDPK*; *2*, purified WbPK. M_rs of the standard proteins are given in the left margin.

Protein kinase assay and definition of protein kinase unit

Protein kinase assay was routinely carried out by the P81 paper (Whatman) binding method in duplicates [16]. The reaction mixture contained 50 mM Tris.HCl (pH-7.5), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.05 mM orthovanadate, BSA (0.5 mg ml^{-1}), 0.05 mM [γ-^{32}P]ATP (500–2000 cpm $pmol^{-1}$) and 0.05 mM peptide substrate (MLC-peptide or syntide 2) or histone H1 (0.16 $mg.ml^{-1}$) in a total vol. of 50 μl. The reaction was initiated by the addition of 0.5–1.0 μg of enzyme in 10 μl and the incubation was at 25° for a fixed time within the linear range. In the case of assays using peptide substrates, the reaction was stopped by the addition of glacial HOAc to a final concn of 45%. Portions of the acidified mixture were spotted on P81 paper strips (2 × 2 cm) which were washed × 3–4 in 15% HOAc and then in 90% EtOH, dried and counted in a liquid scintillation counter. For protein substrates, e.g. H1, the reaction was stopped by spotting a fixed portion of the reaction mixture on P81 paper strips pre-soaked in 10% TCA. They were then thoroughly washed with 20 mM PPi in 5% TCA and in 90% EtOH before drying and counting in a liquid scintillation counter.

Alternatively, the reaction was stopped by adding SDS-sample buffer and the mixture boiled and subjected to SDS–PAGE (20% or 12% acrylamide), followed by autoradiography. The phosphorylated protein band was excised from the gel slab and [^{32}P] incorporation determined by liquid scintillation counting, as described earlier [12]. The specific activity of the protein kinase was calculated from the average value of duplicate assays.

Enzyme unit

One unit of protein kinase activity is defined as the amount of enzyme which catalyses the transfer of 1 nmol of phosphate per min from ATP to its protein/peptide substrate, under the conditions of assay.

Phosphoamino acid analysis

Histone H1 is phosphorylated using WbPK and [γ-^{32}P]ATP, as described above. The reaction is stopped by the addition of 5 × SDS-sample buffer and boiling the mixture for 5 min. The boiled sample was subjected to 12% SDS–PAGE and the fractionated proteins were transferred to PVDF membrane (Amersham). The phosphorylated H1 band was identified by autoradiograpy and excised from the membrane. It was then subjected to hydrolysis in 6 M HCl at 110° for 4 h and the hydrolysate mixed with standard phosphoamino acids and subjected to electrophoresis on thin layer cellulose sheets (Sigma) at 450 V for 90 min using a pyridine-HOAc-H_2O (1 : 10 : 189) buffer

Table 4. Comparison of the kinetic properties of WbPK and WbCDPK. The kinetic parameters were obtained from the Lineweaver-Burk double reciprocal plots. [^{32}P] incorporated in the peptide and protein substrates were determined by the gel assay method [12]. After subjecting the reaction products to SDS–PAGE, the radioactive bands were sliced from the dried gel and quantitated in a liquid scintillation counter

	K_m		V_{max} (nmol min^{-1} mg^{-1})	
Substrate	WbPK	WbCDPK*	WbPK	WbCDPK*
Histone H1	0.035 mg ml^{-1}	0.07 mg ml^{-1}	5.9	1.8
Syntide-2	12.0 μM	7.8 μM	31.2	6.7
MLC-peptide	27.0 μM	14.3 μM	25.0	4.3
ATP	6.8 μM	16.4 μM	26.5	4.1

* The K_m and V_{max} values of WbCDPK were taken from the earlier report [12].

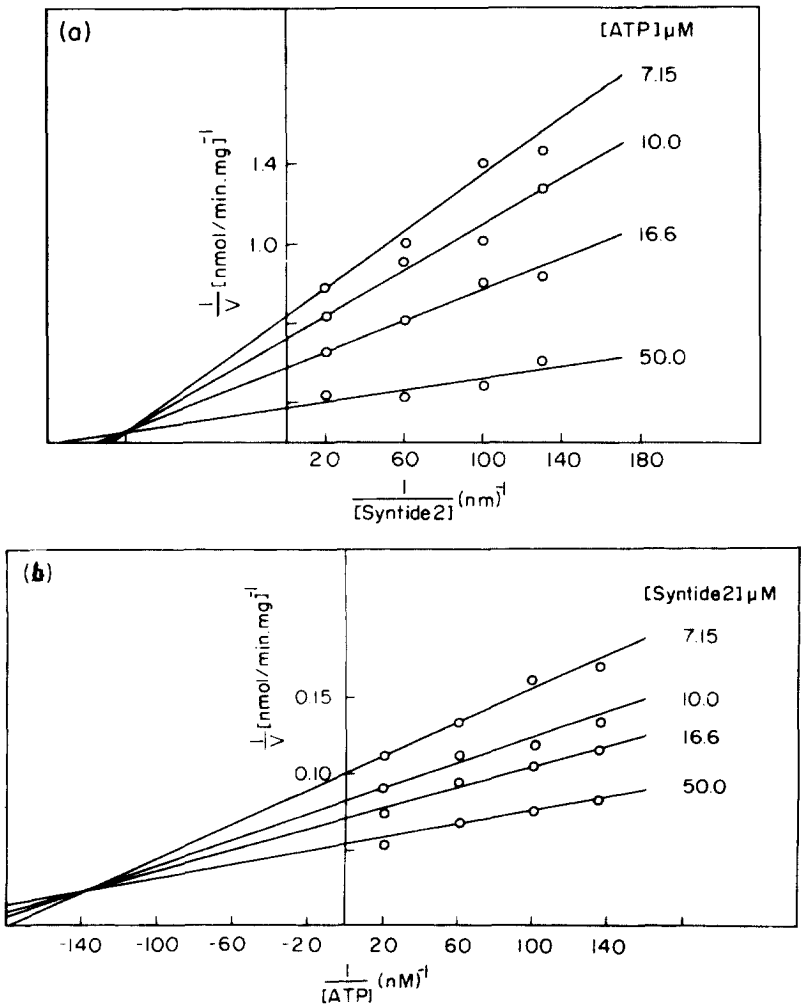

Fig. 7. Kinetic analysis with WbPK. The protein kinase was assayed for 30 s in the presence of different concentrations of syntide 2 and [γ-^{32}P]ATP with 0.5 μg of enzyme and the ^{32}P incorporation was determined by the alternative assay from slab gels as described in the Experimental section. **a.** Lineweaver-Burk plot of 1/v versus 1/[syntide 2] at different fixed concentrations of [γ-^{32}P]ATP, as indicated. **b.** Lineweaver-Burk plot of 1/v versus 1/[ATP] at different fixed concentrations of syntide 2, as indicated.

system [17]. The phosphoamino acids were identified by autoradiography and ninhydrin staining.

Other methods

The M_r of the native enzyme was determined by analytical gel filtration [18] and sucrose density gradient centrifugation [19]. Protein was determined by the Coomassie Blue-dye binding method of ref. [18], with BSA as the standard. SDS–PAGE was done as described by in ref. [21] and for immunoblot analysis, the method of ref. [22] was followed.

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