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Research article

Biochemical characterization of a recombinant *Swainsona canescens* calcium-dependent protein kinase (ScCPK1)

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ABSTRACT

Calcium-dependent protein kinases (CPKs) constitute a unique family of kinases involved in many physiological responses in plants. Biochemical and kinetic properties of a recombinant *Swainsona canescens* calcium-dependent protein kinase (ScCPK1) were examined in this study. The optimum pH and temperature for activity were pH 7.5 and 37 °C, respectively. Substrate phosphorylation activity of ScCPK1 was calmodulin (CaM) independent. Yet CaM antagonists, W7 [N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide] and calmidazolium inhibited the activity with IC_{50} values of 750 nM and 350 μM, respectively. Both serine and threonine residues were found to be phosphorylated in auto-phosphorylated ScCPK1 and in histone III-S phosphorylated by ScCPK1. The [Ca^{2+}] for half maximal activity ($K_{0.5}$) was found to be 0.4 μM for ScCPK1 with histone III-S as substrate. Kinetic analysis showed that K_M of ScCPK1 for histone III-S was 4.8 μM. These data suggest that ScCPK1 is a functional Ser/Thr kinase, regulated by calcium, and may have a role in Ca^{2+}-mediated signaling in *S. canescens*.

1. Introduction

Calcium-dependent protein kinases (CPKs) are predominant calcium sensors in plants shown to be involved in a myriad of physiological responses [1–4]. CPKs are Ser/Thr family of protein kinases typically made up of five domains with an N-terminal variable domain followed by catalytic protein kinase domain, an autoinhibitory/junction domain, a regulatory calmodulin-like domain (CaM-LD) and a C-terminal domain of variable length [1,2,5–7]. The CPKs are unique due to the presence of CaM-LD which couples the calcium sensor directly to its responder (kinase domain). From an evolutionary standpoint, it has been suggested that the genes encoding CPKs might have evolved by the fusion of a gene encoding the catalytic/autoinhibitory domain of a Ca^{2+}/CaM-dependent protein kinase and a gene encoding a CaM-like protein [8,9]. However, CPKs are confined mainly to the plant kingdom and few protozoans *viz Plasmodium* [10], *Toxoplasma gondii* [11] and *Paramecium* [12]. However, CPKs are notably absent in the eukaryotic genome of yeast (*Saccharomyces cerevisiae*), nematodes [13], fruitflies (*Drosophila melanogaster*) [14]; and humans (International human genome sequencing consortium 2001) [15]. Several CPKs have been isolated from a variety of plant species and their role in plant physiology have been reviewed extensively [1–4]. Over 30 CPK isoforms were found in *Arabidopsis* [1,2] and rice [16,17].There has been considerable progress in plant CPK studies for identifying new isoforms and cloning corresponding genes to investigate their specific roles in signal transduction cascades involving plant growth and development, and various stress responses.

Several studies have been carried out to correlate the effect of calcium on secondary metabolite production [18–20]. Being calcium sensors in plants, CPKs may play a role in secondary metabolite production. It has been suggested that CPKs may be closely associated with production of ginsenoside Rb1 by *Panax notoginseng* cells [20]. Moreover, the involvement of the general Ser/Thr protein kinases was also reported in elicitor-induced production of anthraquinone and saponin [21,22]. Our laboratory has demonstrated the involvement of CPK in UV-B induced accumulation of catharanthine in *Catharanthus roseus* cell suspension cultures [23]. It has been observed that heterologous expression of *Arabidopsis* CPK gene in *Rubia cordifolia* cells induced anthraquinone biosynthesis [24]. Recently we have isolated and characterized a cDNA encoding a calcium-dependent protein kinase (ScCPK1) from *Swainsona canescens*; with a view to investigate the role of ScCPK in the biosynthesis of swainsonine, a pharmaceutically important anti-cancer secondary metabolite produced by this

Abbreviations: CDPK, calcium-dependent protein kinase; ScCPK1, *Swainsona canescens* calcium-dependent protein kinase; CaM, calmodulin.

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Australian herb. Here we report biochemical and kinetic properties of recombinant ScCPK1 from *Swainsona canescens.*

2. Results

2.1. Biochemical properties of ScCPK1

2.1.1. Calcium ion dependence

Substrate phosphorylation using histone III-S as the substrate was carried out at various Ca^{2+} concentrations (0.1–200 μM) to examine effect of how Ca^{2+} binding affects the activity of the purified recombinant ScCPK1. The free Ca^{2+} concentration in the buffer was calculated using the Maxchelator programme (http://www.stanford.edu/~cpatton/CaEGTA-NIST.htm). There was no detectable kinase activity in the absence of Ca^{2+}. A steep increase in substrate phosphorylation activity was observed with increasing Ca^{2+} concentration with half maximal activity ($K_{0.5}$) at 0.4 μM (Fig. 1).

2.1.2. ScCPK1 activity with respect to pH, temperature and time course

The effect of pH on the substrate phosphorylation reaction of the enzyme was studied using three different buffer systems. ScCPK1 was found to be highly active between pH 7–8, while the activity decreased at pH range on either side, with activity only ~25% at pH 6.0 and 50% at pH 9.0. The enzyme activity persisted even at pH 5.0 and pH 10 (~10%) (Fig. 2 A). The activity steadily increased from 20 °C (20%) up to a maximum at 37 °C thereafter decreased and was negligible at 60 °C (Fig. 2 B). With a small lag, the activity the activity increased linearly upto 8 min with maximum conversion of substrate at 10 min (Fig. 2 C).

2.1.3. Influence of salt concentrations on ScCPK1 activity

The addition of NaCl to the assay buffer had a significant negative effect with substrate phosphorylation activity of ScCPK1 decreased to 25% by 25 mM. Maximum decrease was found at 100 mM NaCl (Fig. 2 D). The ScCPK1 was more sensitive to NaCl than the two chickpea CPKs previously characterized in our laboratory [25]. The ScCPK1 substrate phosphorylation activity required Mg^{2+} with no activity seen in its absence, similar to all other CPKs reported. The optimum concentration of $MgCl_2$ needed for the activity was found at 10 mM with inhibition observed above 10 mM $MgCl_2$ and complete inhibition at 50 mM $MgCl_2$ (Fig. 2 E).

2.1.4. Kinetic properties

To obtain the kinetic properties of ScCPK1, substrate phosphorylation assay was carried out using different concentrations of histone III-S substrate at a fixed concentration of the purified recombinant enzyme. As shown in the Fig. 3 A, the reaction velocity plotted as a function of substrate concentration exhibited a typical hyperbolic saturation curve for ScCPK1. These graphical data were analyzed to calculate kinetic constants, K_M and V_{max}. Lineweaver-Burk plot yielded a K_M histone III-S value of 4.8 μM. The V_{max} values extrapolated from the plots were found to be 4700 pmol min^{-1} mg^{-1} (Fig. 3 B).

2.2. Effect of calmodulin (CaM) and CaM antagonists, calmidazolium and W7, on auto- and substrate phosphorylation activities of ScCPK1

Exogenous calmodulin (0–50 μM) had no effect on substrate phosphorylation activity of ScCPK1 (Fig. 4 A and B). But CaM antagonists, calmidazolium and W7 had inhibitory effect on substrate phosphorylation activity of ScCPK1. Calmidazolium completely inhibited ScCPK1 at 50 μM (Fig. 4 B) while W7 inhibited at much higher concentration (1 mM) (Fig. 4 C). IC_{50} value for calmidazolium was around 750 nM and for W7 it was 350 μM. These observations suggested that ScCPK1 enzyme may have a constitutive CaM-like domain that is impaired in the presence of these CaM antagonists.

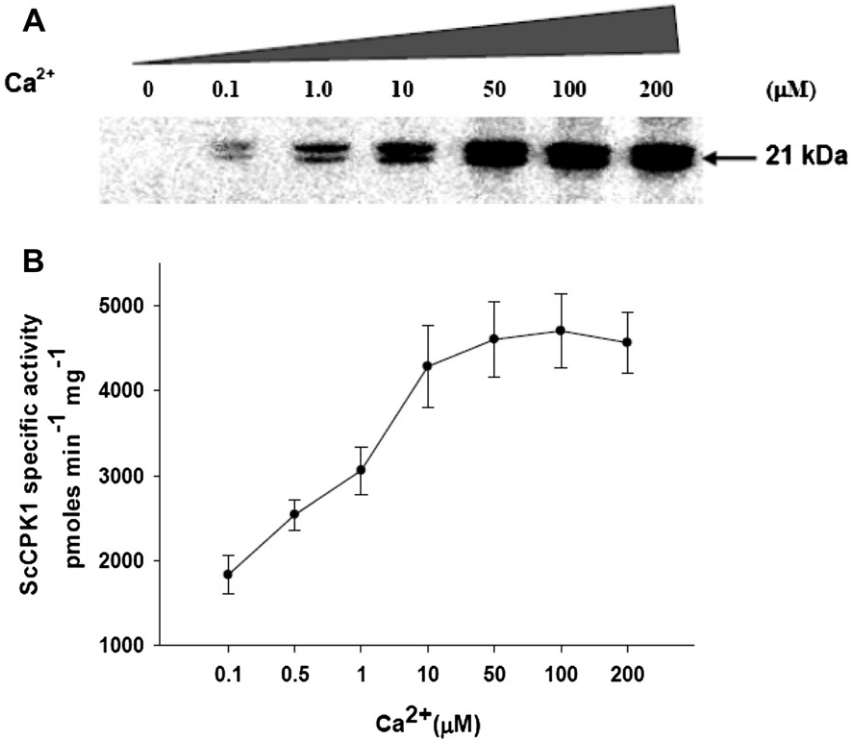

Fig. 1. Effect of Ca^{2+} on the substrate phosphorylation activity of recombinant ScCPK1. A steep increase in substrate phosphorylation activity was observed with increasing Ca^{2+} concentration with half maximal activity ($K_{0.5}$) at 0.4 μM.