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A One-Week Laboratory Practice: Introducing the Students to the Study of Plant Biochemistry and Signal Transduction.

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Abstract: This report describes a one-week laboratory practice for students. An approach to study a Calcium dependent protein kinase (CDPK) involved in signal transduction processes in potato plants, is undertaken. A number of basic biochemical techniques including the partial purification of a protein kinase, protein kinase activity assays, protein determination, SDS-PAGE analysis of phosphorylated proteins and Western blot assays are described.

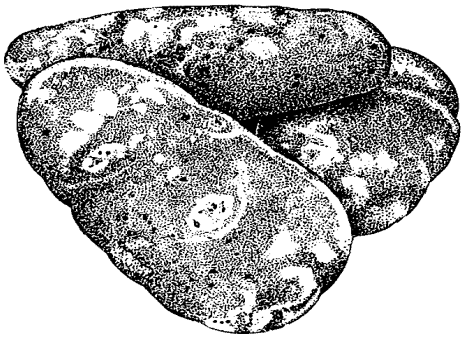
Key Words: plant biochemistry, Calcium dependent protein kinase (CDPK), *Solanum tuberosum*, phosphorylation.

Introduction:

Plants are responsive to external stimuli and endogenous developmental signals that must be recognized and translated into cellular responses. In plants, protein phosphorylation has been implicated in responses to many signals, including light, pathogen invasion, hormones, temperature stress and nutrient deprivation. As might be expected from this diversity of functions, there is a large array of different protein kinases (Stone and Walker, 1995). An estimated 1-3% of functional eukaryotic genes encode protein kinases.

Phosphorylation can have profound effects on enzyme activity and protein interactions. Regulatory phosphorylation can result in inactivation, activation and/or changes in the allosteric properties of the target enzyme. Dephosphorylation of phosphorylated proteins by specific phosphatases offers a mechanism by which the biochemical pathways involving phosphorylation can be deactivated. The steady-state activity of the target enzyme is adjusted over a wide range by positive and/or negative effectors influencing the rates of phosphorylation and dephosphorylation (Cohen, 1992).

Increasing evidence has established calcium as a second messenger in plants (Roberts and Weaver, 1990; Muto, 1992; Poovaiah and Reddy, 1993). The concentration of free cytosolic Ca^{2+} in plant cells can be elevated by various external signals. Such increases in the concentration of free Ca^{2+} is one of the primary events in the transduction of many signals, and can alter biochemical processes in plants by activating particular enzymes.



A multitude of specialized calcium-modulated proteins that serve as receptors for calcium signals have arisen during eukaryotic evolution. Calcium has been shown to affect protein phosphorylation in plants (Budde and Chollet,1988) and a calcium-dependent calmodulin-independent protein kinase (CDPK) was first discovered in soybean (Harmon *et al*., 1987). Later studies demonstrated that this enzyme is widespread in plants and contains a protein kinase catalytic domain and a calcium-binding regulatory domain similar to calmodulin; both regions are separated by a junction domain. This unique molecular structure explains the direct activation of this enzyme by Ca^{2+} and clearly establishes CDPK as the prototype of a new class of protein kinases (Roberts & Harmon, 1992).

Potato plants produce tubers *in vivo* in response to changes in environmental conditions altering the balance of growth regulators. At least three environmental stimuli are believed to be important: daylength, nitrogen nutrition and temperature (Cutter

1978, Ewing 1987). In this practice, a soluble CDPK is purified and partially characterized in potato plants (*Solanum tuberosum*, L) grown *in vitro* under multiplication or tuber inducing conditions.

In our laboratory, this practice is designed for a maximum of 16 students who are divided into four groups. Each member of the team shares responsibilities for a section of the project. Each group works on 10 grams of *in vitro* cultured potato plants (multiplication conditions, M) and 1 gm of one of the four stages of tuber formation (T). These experiments can be performed in five eight hours sessions. Briefly, the project involves the following steps: 1) Partial purification of the CDPK from M plants and obtainment of the crude extracts of each T-inducing condition and preparation of 12% polyacrylamide separating gels with 5% stacking gel; 2) CDPK activity assay of the DEAE-fractions and crude extracts, protein determination of these same fractions, ammonium sulphate precipitation of the active peak and overnight dialysis 3) Phosphorylation of crude extracts with 10 µM [γ^{32}P]ATP (specific activity 500 cpm pmol^{-1}), the reaction mixture may or may not include an exogenous substrate (Histone H1). The samples are electrophoresed and the gels are stained with Coomassie Blue and left overnight for destaining. Another gel is used to resolve untreated DEAE samples and is electrotransferred to nitrocellulose. After blocking, the nitrocellulose is incubated overnight with a polyclonal antibody against CDPK. An enzyme concentration curve is done with the dialyzed peaks to choose the adequate concentration to perform the next day characterization; 4) Characterization of the peak with CDPK activity includes a) $K_{0.5}$ for Calcium, b) assaying different substrates for phosphorylation acceptors, c) Km for syntide and d) Km for ATP. Radioactive gels are dried and exposed; Western blot is revealed and developed with the Renaissance detection system and 5) The X-ray films are revealed. The data from all groups are exposed, exchanged and discussed.

Experimental.

Day 1: Reagents and material required for experiment 1 (Partial purification of the CDPK from M plants and obtaining the crude extracts of each T-inducing condition).

1) Cultures: Micropropagation of virus-free meristematic sprouts of *Solanum tuberosum*, L. var. Spunta is carried out in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 30 g L^{-1} sucrose, and plants are grown in a growth chamber under 16 h light photoperiod at 25°C (multiplication conditions).

Tuberization is induced with MS medium containing 500 mg L^{-1} of 2-chloroethyltrimethyl-ammonium chloride (CCC) and 80 g L^{-1} sucrose. Plants are grown in complete darkness under inducing conditions at 21±2 °C, using stem cuttings and stolons. Four stages of the process of tuber development are defined according to their morphological aspect: stolons, incipient tuber, tuber enlargement and mature tuber.

2) Liquid nitrogen, mortars, glassware, tubes, bottles and adaptors for centrifuges and ultracentrifuges, and cheese-cloth or glass-wool to filter plant debris.

3) Extraction buffer A: 10 mM Tris-HCl, pH 7.5 containing 1 mM $MgCl_2$, 2 mM KCl, 2 mM β-mercaptoethanol, 3 mM EDTA, 1 mM EGTA, 5% (v/v) glycerol, 0.25 M sucrose, 2 % (w/v) polyvinylpolypirrolidone and protease inhibitors (0.1 mM PMSF, 1 mM benzamidine, 2 µg ml^{-1} soybean trypsin inhibitor and 25 U ml^{-1} aprotinin).

4) 40 ml of pre-hydrated DEAE-cellulose (DE-52) in 80 ml of buffer B: 20 mM Tris-HCl pH 7.5; 0.5 mM EGTA; 0.5 mM EDTA and make a slurry. Glass column (1.6 x 12 cm) to be packed with DEAE-celullose (DE-52), *canules* and clamps. NaCl 0.5M and buffer B in excess.

Preparation of extracts and purification procedures: Plants from multiplication conditions (M) or from each tuberization stage are harvested, rinsed with distilled water, weighted and immediately frozen with liquid nitrogen. The samples are ground in a mortar cooled with liquid nitrogen and extracted with Buffer A (1 ml buffer g^{-1} of wet tissue). The suspensions are centrifuged 10 min at 2,500 x g and the supernatant (crude extract) is filtered through glass wool. The pellet, containing cell debris, is discarded.

The supernatant from M plants is centrifuged 15 min at 12,000 x g and further centrifuged 1 h at 105,000 x g obtaining a cytosolic fraction (S100) and a particulate fraction (P100).

In the meantime, the students pack a glass column with DEAE-cellulose (DE-52). This is done by filling the column half way with buffer B and then pouring the slurry of DEAE in the same buffer. As the resin gradually settles at the bottom of the column, open the valve to increase the flow. Keep adding until the slurry settles to the desired height in the column (5 cm).

The resulting supernatant is diluted 1:5 (v:v) in buffer B with protease inhibitors and loaded on the column equilibrated with buffer B. The column is washed with the same buffer (100 ml) and eluted step-wise with buffer B containing increasing concentrations of NaCl 0.1, 0.2, 0.3, 0.4 and 0.5 M (10 ml of each). The fractions (1.5 ml) are collected manually. **All the extraction procedure is done at 4°C.**

Reagents and materials for experiment 2:

1) 30% Acrylamide/1% bis-Acrylamide solution in distilled water.
2) 1.5 M Tris-HCl buffer pH 8.8
3) 1.0M Tris-HCl buffer pH 6.8
4) 10 % SDS v/v.

5) TEMED (N,N,N',N'-tetramethylethylene diamine).
6) 10% ammonium persulfate (freshly prepared)

Each group of students will prepare a 12% polyacrylamide separating gel with a 5% stacking gel, that will be used during the following days. SDS-PAGE will be performed according to Laemmli (1970).

Protocol for making the 12% polyacrylamide separating gel with a 5% stacking gel:

1) Mix 4 ml of acrylamide/bis-acrylamide solution (30:1) with 2.5 ml of Tris-HCl buffer 1.5M, pH 8.8, 0.1 ml of SDS 10 % V/V and 3.3 ml of distilled deionized water. 2) Add and gently mix 0.1 ml of 10% APS and 4 µl TEMED and pour immediately into the glass plate sandwich assembled using glass plates separated with 1.5 mm Teflon spacers. 3) Overlay with water and allow the gel to polymerize for 30 min at room temperature. 4) Remove water layer, and prepare a 5% stacking gel by mixing 0.5 ml of acrylamide/bis-acrylamide solution with 0.38 ml of Tris-HCl buffer 1.0M, pH 6.8, 0.03 ml of SDS 10 % V/V and 2.1 ml of distilled deionized water. Add 0.03 ml of 10% APS and 3 µl TEMED, pour the solution over the separating gel and insert the Teflon comb. The stacking gel polymerizes in 20 min. The gels should be kept wrapped with foil at 4 °C until needed.

Day 2:

Reagents and materials required for experiment 3 (Protein kinase activity assays):

1) 1 M Tris-HCl buffer, pH 7.5
2) 1 M $MgCl_2$
3) 1M β-mercaptoethanol
4) 10 mM cold ATP and ATP [γ ^{32}P] (3000 Ci/mmol)
5) 2 mM Syntide-2
6) 10 mM $CaCl_2$
7) 10 mM EGTA
8) Racks, pipettes and tips, test tubes, gyratory water bath shaker, timers, gloves.
9) P81 chromatographic paper (phospho-cellulose), scissors, forceps.
10) 75 mM ortho-phosphoric acid
11) Acetone
12) Oven
13) Omnifluor -Toluene (scintillating reagent), scintillation plastic and glass vials with screw caps.
14) Liquid Scintillation Counter.
15) Geiger-Muller monitor.

Protocol for Experiment 3: Aliquots of the different fractions: crude extracts from the 4 tuberization stages, S100 and P100 and every two fractions obtained from the DEAE-cellulose columns are assayed in a reaction mixture containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 µM [γ^{32}P]ATP (specific activity 100 cpm pmol^{-1}), 10 mM β-mercaptoethanol and 25 µM Syntide-2 with the addition of 1 mM EGTA or 1 mM $CaCl_2$, in a final volume of 0.06 ml.

Condition 1: EGTA. 20 µl of each sample (enzyme source) + 14 µl H_2O+ 6 µl **EGTA** 10 mM + 20 µl reaction mixture

Condition 2: Ca^{2+}. 20 µl of each sample (enzyme source) + 14 µl H_2O + 6 µl Ca^{2+} 10 mM + 20 µl reaction mixture

A negative control is performed for each condition without the addition of enzyme. This value will be subtracted from the other data obtained.

Reactions are initiated by the addition of the reaction mixture containing [γ^{32}P]ATP. Assays are performed at 30° C for 10 min and stopped by spotting an aliquot of each assay mixture (40 µl) on 2x2 cm squares of phosphocellulose paper P81, that are immediately immersed in ice-cold 75 mM phosphoric acid for 15 min. Another two 7 min washes are performed with 75 mM phosphoric acid. In order to dry the papers, they are immersed in acetone and oven dried. It is very important to measure the total cpm of the reaction mixture. In order to do this a 5 µl aliquot of the mixture is spotted onto a phosphocellulose paper and dried without washing it with 75 mM phosphoric acid. Radioactivity is determined using an omnifluor-toluene scintillation mixture.

Data Processing:

The enzyme's activity is expressed as pmoles of ^{32}P incorporated min^{-1}. Plot data vs fraction number. A typical DEAE elution profile is shown in figure 1.

The data in cpm should be converted to pmoles of ^{32}P incorporated min^{-1}, applying the following formula:

$$\text{Enzymatic activity (EA)} = \frac{\Delta\text{cpm} \times}{\text{pmoles ATP} \times 60\ \mu\text{l}}$$

$$40\ \mu\text{l} \times 10\ \text{min} \times \text{total cpm}$$

Δcpm = cpm - cpm negative control

pmoles ATP = cold ATP in the mixture = 3000 pmoles

Specific activity = EA .mg^{-1}

Results of the crude extracts specific activity are shown in figure 2.

Reagents and materials required for experiment 4 (Protein Determination):

1) Modified Bradford Reagent: Dissolve 40 mg of Coomassie Blue G250 in 50 ml of ethanol, 100 ml of phosphoric acid 85 % and 850 ml of water.
2) bovine serum albumin (BSA)1 mg/ml.
3) 96-well ELISA plates.
4) ELISA reader for optical determination at 660 nm.

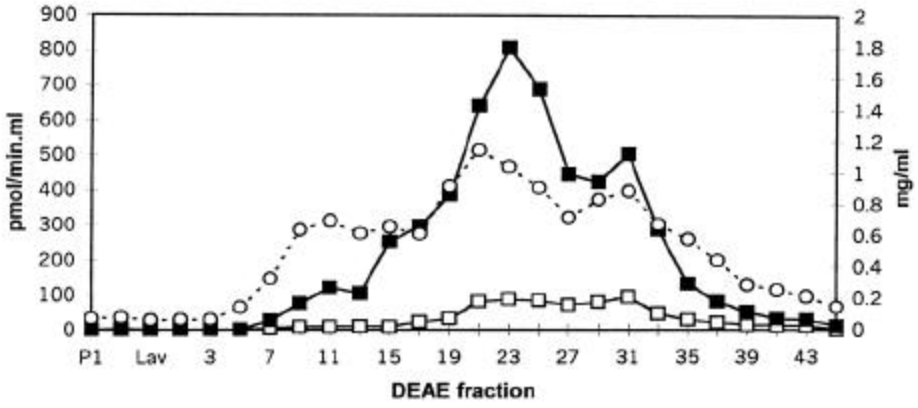


Figure 1: *Purification of the potato CDPK. Elution profile of the DEAE cellulose column. CDPK activity was assayed with 1 mM EGTA (❑) or 1 mM $CaCl_2$ (■). Proteins, (❍).*

Protocol:

Protein contents of the different fractions: crude extracts from the 4 tuberization stages, S100 and P100 and every two fractions obtained from the DEAE-cellulose columns, are determined according to the method of Bradford (1976) using BSA as standard.

An aliquot from each sample (20 µl of the DEAE fractions and S100 or 5 µl of the crude extracts and pellets) is diluted with water to a final volume of 50 µl and mixed with 300 µl of Bradford reagent. After 2 min, 150 µl of each mixture is loaded in an ELISA plate. Read optical density at 660 nm in an ELISA reader. A negative control is carried out with 50 µl of water and another one with 50 µl of extraction buffer.

A calibration curve is performed using 2 µg, 5 µg, 10 µg and 12.5 µg of BSA dissolved in 50 µl of water. Standards are treated in the same way as the samples. Plot data vs fraction number. A typical profile is shown in figure 1.

Reagents and materials required for experiment 5 (Protein precipitation)

1) Ammonium sulphate salt.
2) Bottles for centrifuge and refrigerated centrifuge.
3) Buffer B (2 l)
4) Magnetic stirrer, dialysis bags and glassware

Protocol: The DEAE fractions with CDPK activity are pooled and precipitated with 70% ammonium sulphate in order to concentrate the sample. This salt is commonly employed on account of its large solubility in water and absence of harmful effects on most enzymes.

After measuring the exact volume of the pooled fractions, an adequate amount of salt (375 g/l) is very slowly added while stirring, care should be taken that all the crystals are dissolved. The precipitation is carried out during 20 min in an ice cold bath and this solution is then centrifuged at 15.000 rpm during another 20 min at 4 °C. The supernatant is discarded and the pellet is resuspended in 2 ml of buffer B. The precipitated fractions are overnight dialyzed against 2 l of buffer B.

Day 3.

Reagents and materials required for experiments 6, 7 and 8 (phosphorylation assays, SDS-PAGE, Western blot).

1) 12% polyacrylamide separating gels with a 5% stacking gel
2) Radioactive mix: 5 µM [γ^{32}P]ATP (10,000 cpm $pmol^{-1}$) in 20 mM Tris-HCl pH 7.5, 10 mM β-mercaptoethanol and 10 mM $MgCl_2$.
3) 10 mM EGTA or 10 mM $CaCl_2$.
4) Histone H1 1 mg /ml.
5) 5X SDS sample buffer (vol 20 ml): 2 g SDS, 1.54 g DTT, 8 ml de Tris-HCl pH 6.8. Dissolve at 50 ºC and add 10 ml of glycerol and 0.002 % p/v of bromophenol blue.
5) Running Buffer: Tris 3.03 g/l, Glycine 4.4 g/l, SDS 0.1 % v/v.
6) Electrophoresis apparatus (Bio-Rad Mini-Protean II™).
7) Power supply.
8) Staining solution: 45 % v/v methanol, 10 % v/v acetic acid, 0.25 %p/v Coomassie Brilliant Blue.
9) Destaining solution: 45 % v/v methanol, 10 % v/v acetic acid in water.
10) Transfer buffer: Tris 3.03 g/l, Glycine 14.4 g/l, 20 % methanol.
11) Minitrans blot Electrophoretic Transfer cell.
12) Blocking solution: TBS 1X with 3 % skimmed milk.

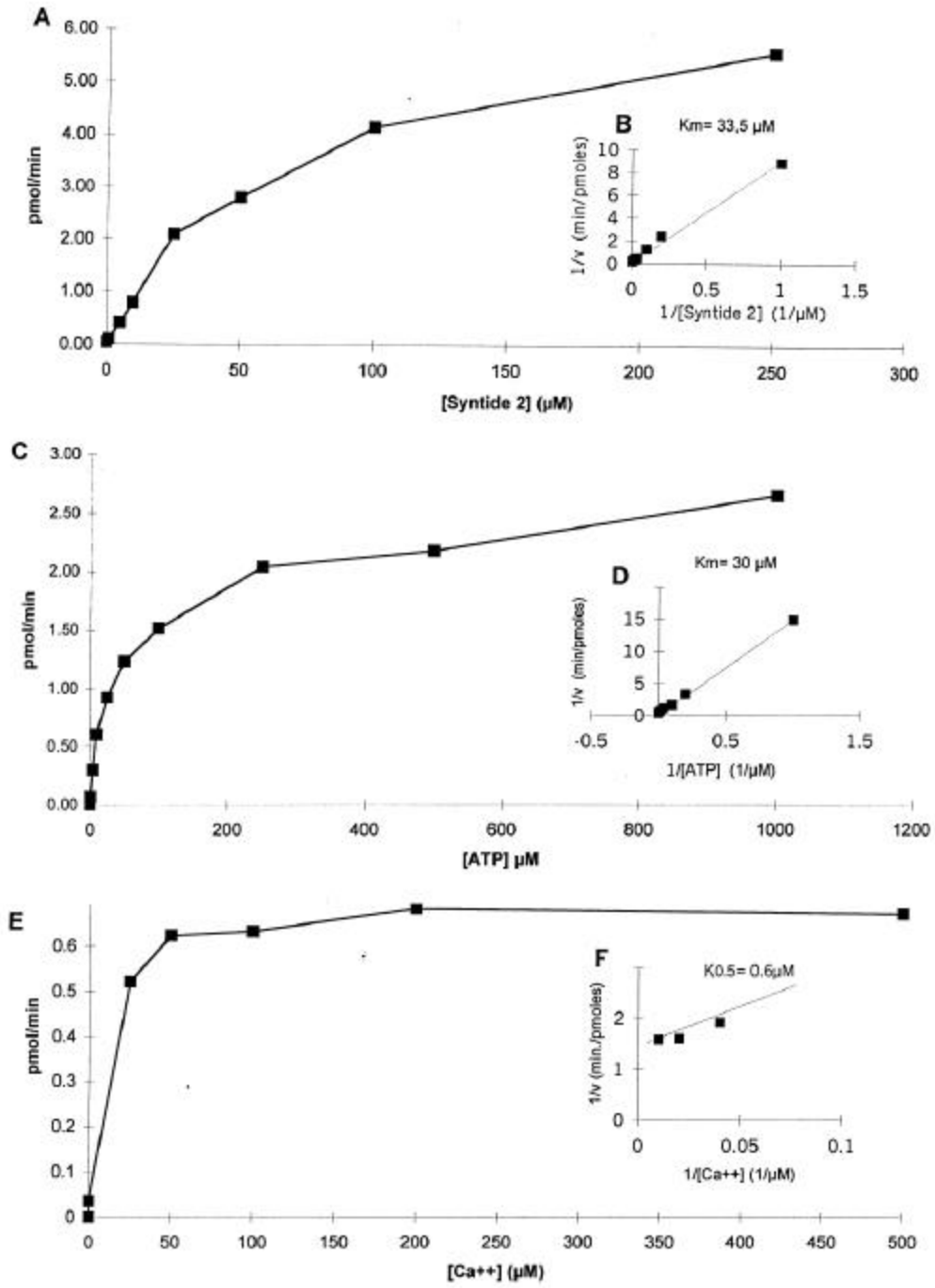


Figure 2 ***A:*** *Km for syntide-2. A concentration curve was performed using increasing syntide-2 concentrations. The assay was performed in the presence of 1 mM Ca^{2+}. Reactions were initiated by the addition of the reaction mixture prepared without substrate and containing [g^{32}P]ATP. The plot shows enzymatic activity vs syntide-2 concentration and the inset* ***B*** *shows the double reciprocal plot (1/v vs 1/[S]).* ***C:*** *Km for ATP. A concentration curve was performed using increasing ATP concentrations. The assay is performed in the presence of 1 mM Ca^{2+}. Reactions are initiated by the addition of the reaction mixture prepared without cold ATP but containing [g^{32}P]ATP. The plot shows enzymatic activity vs ATP concentration and the inset* ***D*** *shows the double reciprocal plot (1/v vs 1/[ATP]).* ***E:*** *$K_{0.5}$ for Calcium. A calcium concentration curve was performed using increasing calcium concentrations. The activity in the absence of calcium is assayed in the presence of 1 and 5 mM EGTA. Reactions are initiated by the addition of the reaction mixture prepared without cold ATP but containing [g^{32}P]ATP. The plot shows enzymatic activity vs Ca^{2+} concentration and the inset* ***F*** *shows the double reciprocal plot (1/v vs 1/[Ca^{2+}]).*

Protocol:

Protein contents of the different fractions: crude extracts from the 4 tuberization stages, S100 and P100 and every two fractions obtained from the DEAE-cellulose columns, are determined according to the method of Bradford (1976) using BSA as standard.

An aliquot from each sample (20 µl of the DEAE fractions and S100 or 5 µl of the crude extracts and pellets) is diluted with water to a final volume of 50 µl and mixed with 300 µl of Bradford reagent. After 2 min, 150 µl of each mixture is loaded in an ELISA plate. Read optical density at 660 nm in an ELISA reader. A negative control is carried out with 50 µl of water and another one with 50 µl of extraction buffer.

A calibration curve is performed using 2 µg, 5 µg, 10 µg and 12,5 µg of BSA dissolved in 50 µl of water. Standards are treated in the same way as the samples. Plot data vs fraction number. A typical profile is shown in figure 1.

Reagents and Materials Required for Experiment 5 (Protein Precipitation)

Ammonium sulphate salt.
Bottles for centrifuge and refrigerated centrifuge.
Buffer B (2 l)
Magnetic stirrer, dialysis bags and glassware

Protocol: The DEAE fractions with CDPK activity are pooled and precipitated with 70% ammonium sulphate in order to concentrate the sample. This salt is commonly employed on account of its large solubility in water and absence of harmful effects on most enzymes.

After measuring the exact volume of the pooled fractions, an adequate amount of salt (375 g/l) is very slowly added while stirring, care should be taken that all the crystals are dissolved. The precipitation is carried out during 20 min in an ice cold bath and this solution is then centrifuged at 15,000 rpm during another 20 min at 4 °C. The supernatant is discarded and the pellet is resuspended in 2 ml of buffer B. The precipitated fractions are overnight dialyzed against 2 l of buffer B.

Day 3.

Reagents and materials required for experiments 6, 7 and 8 (phosphorylation assays, SDS-PAGE, Western blot).

12% polyacrylamide separating gels with a 5% stacking gel
Radioactive mix: 5 µM [γ^{32}P]ATP (10,000 cpm pmol^{-1}) in 20 mM Tris-HCl pH 7.5, 10 mM β-mercaptoethanol and 10 mM $MgCl_2$.
10 mM EGTA or 10 mM $CaCl_2$.
Histone H1 1 mg /ml.
5X SDS sample buffer (vol 20 ml): 2 g SDS, 1.54 g DTT, 8 ml de Tris-HCl pH 6.8. Dissolve at 50 ºC and add 10 ml of glycerol and 0.002 % p/v of bromophenol blue.
Running Buffer: Tris 3.03 g/l, Glycine 4.4 g/l, SDS 0.1 % v/v.
Electrophoresis apparatus (Bio-Rad Mini-Protean II™).
Power supply.
Staining solution: 45 % v/v methanol, 10 % v/v acetic acid, 0.25 %p/v Coomassie Brilliant Blue.
Destaining solution: 45 % v/v methanol, 10 % v/v acetic acid in water.
Transfer buffer: Tris 3.03 g/l, Glycine 14.4 g/l, 20 % methanol
Minitrans blot Electrophoretic Transfer cell
Blocking solution: TBS 1X with 3 % skimmed milk

Protocol for Endogenous Phosphorylation Assays and Histone Phosphorylation:

Experiment 6: Endogenous phosphorylation: Crude extracts of each tuberization stage (100 µg of protein) or S100 and P100 from the plants grown under multiplication conditions (100 µg protein) are incubated 5 min at 30°C with the radioactive mixture in a final volume of 40 µl.

Experiment 7: Histone phosphorylation: 100 µg of S100 or 50 µg of the concentrated DEAE-fractions are incubated with the radioactive mixture containing 0.1 mg/ml of histone H1 mixture in a final volume of 40 µl.

Assays of endogenous phosphorylation and histone phosphorylation are done in two conditions:

Condition 1: 100 µg of each sample + 4 µl 10 mM EGTA + 10 µl reaction mixture

Condition 2: 100 µg of each sample + 4 µl 10 mM **Ca^{2+}** + 10 µl reaction mixture. Reactions are stopped by the addition of 10 µl cracking buffer 5X and boiled for 5 min.

Experiment 8: Western blot analysis: Aliquots from the DEAE-cellulose columns (50 µg) are mixed with cracking buffer and boiled. In all cases, prestained SDS-PAGE standards from GIBCO-BRL are used as molecular weight markers.

Prepare the electrophoresis unit filling both upper and lower chamber with the running buffer. Load the different samples. Connect to the power supply and run the gels at 20 mA for the stacking gel and 35mA for the separating gel, until the bromophenol blue dye reaches the bottom of the gel. Total run time is usually 2 hours. Carefully disassemble the gel and stain the radioactive gels with Coomassie Brilliant Blue solution for 30 min and destain overnight.

The polypeptide transfer from polyacrylamide to nitrocellulose membranes is carried out using the transfer cell during 1 hour at 80 volts. Cooling is

provided by a self-contained Bio-Ice unit which absorbs heat generated during transfer. Blots are incubated 2 hours in blocking solution at room temperature and are then incubated overnight at 4°C with affinity purified polyclonal antibodies directed against the calmodulin-like-domain (CLD) of soybean α CDPK (Bachmann *et al*. 1996). All the procedure should be done under continuous agitation.

Experiment 9: Enzyme Concentration Curve:

The reagents and materials required for Experiment 9 are the same used in experiment 3.

In order to determine the optimal conditions to characterize some kinetic parameters of the CDPK an enzyme concentration curve is performed. The concentrated CDPK is used as enzyme source and the assay is done in two conditions. Protein determination of this fraction is done according to Bradford (1976).

Condition1 : 2, 5, 10, 20 and 30 µl of enzyme + 6 µl of 10 mM EGTA + H_2O to reach 40 µl.

Condition 2 : 2, 5, 10, 20 and 30 µl of enzyme + 6 µl of 10 mM Ca^{2+} + H_2O to reach 40 µl. The reaction mixture (20 µl) is the same used in experiment 3.

Plot Enzyme Activity vs. µg protein and choose the appropriate concentration to measure initial velocity.

Day 4:

The SDS-PAGE and Western blot analysis are continued.

Radioactive gels: gels are vacuum dried during two hours at 70°C in a gel dryer and exposed overnight to x-ray films with an amplifying screen at -70°C. Autoradiographies are revealed next morning. An example of histone phosphorylation using the cytosolic fraction as enzymatic source is shown in figure 3A. An assay of endogenous phosphorylation performed with the crude extracts of the different stages can be observed in figure 4.

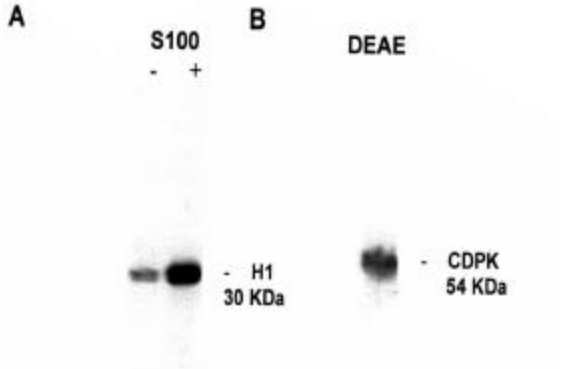


Figure 3 ***A:*** *Histone phosphorylation by soluble extracts from potato plants grown under multiplication conditions (50mg protein in each lane). Incubations were carried out with 5 mM [g^{32}P] ATP and 1 mg ml^{-1} Histone H IIIS and 1 mM Ca^{2+} (+) or 1 mM EGTA (-). Samples were analyzed by SDS-PAGE.* ***B:*** *Western blot analysis of the DEAE fraction (100µg protein) with polyclonal antibodies against the regulatory domain of the αCDPK from soybean.*

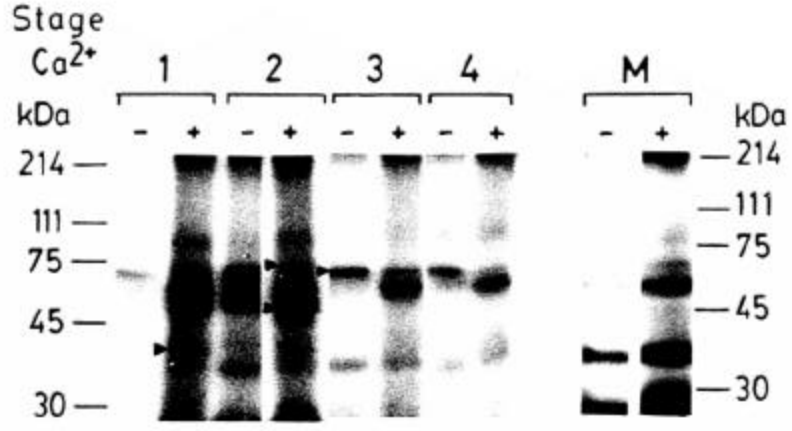


Figure 4: *Endogenous phosphorylation in the different stages of tuber development (1, 2, 3, 4) and in plants grown under multiplication conditions (M). Crude extracts of the different stages (1 and 2, 50 mg of protein; 3 and 4, 100 mg of protein) and of M (100 mg of protein) were incubated with 5 mM [g^{32}P] ATP (5000 cpm $pmol^{-1}$) and 1 mM Ca^{2+}. Arrows indicate stage-specific phosphorylated or dephosphorylated peptides. Molecular weight markers are indicated.*

Western blot: The blot is washed four times with TBS-Tween™ (0.5%), 5 min each wash, and is then incubated at room temperature during 1 hour with a goat anti-rabbit second antibody (1:2500 in TBS-Tween™ (0,5%) with 5% skimmed milk).

The blot is washed three times with TBS-Tween™ (0.5%) (5 min each wash) and is developed with Renaissance, Western Blot Chemiluminescence reagent from NEN according to the manufacturer's procedure. A Western Blot performed with the DEAE fraction of potato plants is shown in Figure 3B.

Experiment 10: Kinetic parameters of the enzyme are determined:

a) $K_{0.5}$ for Ca^{2+}; b) different substrates are assayed as phosphorylation acceptors; c) Km for syntide-2; d) Km for ATP.

Reagents and materials required are the same used in experiment 3.

Each group will determine one parameter and the results will then be shared in common. In all cases the enzyme source will be the precipitated fraction at the selected concentration to work at initial velocity.

It is a sound principle in enzyme work to determine the effect on the initial velocity of varying one factor at a time while all the others are held constant.

a) $K_{0.5}$ for Calcium:

CDPK activity is dependent on the presence of micromolar concentrations of Ca^{2+}. A calcium concentration curve will be prepared utilizing the following concentrations: 0.001 mM, 0.005 mM, 0.01 mM, 0.025 mM, 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM and 1 mM. The reaction mixture and the assay conditions are the same used in experiment 3.

The activity in the absence of calcium is assayed in the presence of 1 and 5 mM EGTA.

Plot enzymatic activity vs calcium concentration and the double reciprocal plot (1/v vs 1/ Ca^{2+}) in order to determine the K_a for Ca^{2+}. An example of this plot is shown in figure 2C. The K_a for Ca^{2+} is 0.6µM.

b) Acceptors of phosphorylation:

The CDPK activity is assayed with different exogenous acceptors of phosphorylation in the presence or absence of Ca^{2+}.

Proteins: Protamine, Histone 2AS and Histone 3S (H1)are used in a final concentration of 1 mg/ml.

Synthetic peptides: Kemptide, Syntide-2, GS, CDPKS, and CDPKS analogue are used in a final concentration of 25 µM.

Condition 1: enzyme source+ substrate + 1 mM EGTA + H_2O until 40 µl.

Condition 2: enzyme source+ substrate + 1 mM Ca^{2+} + H_2O until 40 µl.

Reactions are initiated by the addition of the reaction mixture prepared without substrate and containing [γ^{32}P]ATP. A negative control without enzyme is performed for each substrate. Assays are performed at 30° C for 10 min and stopped as indicated for experiment 3.

Data should be expressed as pmoles of ^{32}P incorporated min^{-1} mg^{-1}.

As shown in Table 1, Histone H1 is the best exogenous protein substrate; however, synthetic peptides that mimics the phosphorylation site of the glycogen synthase enzyme, such as GS, Syntide-2 and CDPKS analogue, are 5 to 8 fold better than Histone H1. The highest activation (14.3 times) is achieved using Syntide-2 as substrate.

c) K_m for Syntide-2:

In order to determine the Km for syntide-2, a concentration curve will be performed. Increasing syntide-2 concentrations are added in each tube: 2.5 µM, 5 µM, 10 µM, 25 µM , 50 µM, 100 µM, and 250 µM. The assay is performed in the presence of 1 mM Ca^{2+}.

Reactions are initiated by the addition of the reaction mixture prepared without cold ATP but containing [γ^{32}P]ATP. A negative control without enzyme is performed. Assays are performed at 30° C for 10 min and stopped as indicated for experiment 3.

Plot enzymatic activity vs syntide-2 concentration and the double reciprocal plot (1/v vs 1/[S]). An example of this plot is shown in figure 2a. The Km value for synthide-2 is 33 ± 5 µM.

d) Km for ATP:

In order to determine the Km for ATP, a concentration curve will be performed. Increasing ATP concentrations are added in each tube: 2.5 µM, 5 µM, 10 µM, 25 µM, 50 µM, 100 µM, 250 µM, 500 µM and 1 mM (final concentrations). The assay is performed in the presence of 1 mM Ca^{2+}.

Reactions are initiated by the addition of the reaction mixture prepared without cold ATP but containing [γ^{32}P]ATP. A negative control without enzyme is performed. Assays are performed at 30° C for 10 min and stopped as indicated for experiment 3.

Plot enzymatic activity vs ATP concentration and the double reciprocal plot (1/v vs 1/[ATP]). An example of this plot is shown in figure 2B. The K_m value for ATP is 30 ± 5 µM.

Day 5:

Discussion of results: All data are analyzed and shared in common. Each group informs the results obtained and analyzes each experiment considering the drawbacks and problems that arose. General conclusions should be drawn and the students must compare their results with those reported in the literature.

	Final Concentration	CDPK Activity		Activation
		EGTA	Ca2+	
		%		times
Proteins				
Histone III S	1 mg/ml	34.0	100.0	2.9
Histone IIA S	1 mg/ml	4.0	8.0	2.0
Protamine	1 mg/ml	3.5	10.0	2.8
Synthetic Peptides				
GS	25 μM	95.0	880.0	9.2
Syntide-2	25 μM	44.0	632.0	14.3
CDPKS analogue	25 μM	39.0	520.0	13.3
MBP	100 μM	31.0	57.0	1.8
CDPKS	25 μM	6.0	37.0	6.2
Kemptide	25 μM	7.0	28.0	4.0

100% activity = 180 pmol ^{32}P/ min mg

Table 1: *Substrate specificity of potato CDPK. All assays were carried out with the DEAE preparations. CDPK was assayed under standard conditions with 1mM Ca^{2+} or 1mM EGTA. Protein kinase activity is presented as percent of control rate in the presence of H IIIS and 1mM Ca^{2+}. 100% = 32 pmol $min^{-1}mg^{-1}$.*

Discussion.

These experiments require standard equipment available in universities or institutes with programs in biochemistry and include a spectrophotometer, micropipettes, gyratory water bath shaker, electrophoresis apparatus, transfer cell, gel dryer, refrigerated ultracentrifuges and a β-Counter. In addition, it is necessary to have the reagents used in the protein kinase activity assays. Usually, these reagents can be purchased from commercial sources. The plant material used in this practice is prepared in advance by the instructors in charge.

The experiments performed in this practice give the students a general view on how to begin a purification protocol of a soluble enzyme. Only a preliminary characterization of the enzyme is undertaken but it introduces the student to some of the conditions that should be taken into account during enzymatic characterization. In plants, protein phosphorylation has been observed *in vivo,* and protein kinase activity has been demonstrated *in vitro*. In addition, this practice focuses on the comparison of a regulated protein kinase activity between plants grown under different conditions, in order to approach the idea of how plants respond to external stimuli.

It is our experience that this practice is feasible and positive results are obtained (MacIntosh *et al*, 1996 and Ulloa *et al*, 1997). The fact that each group is responsible for an area of work, adds a special interest *in* performing the experiments carefully. Primarily, the methods used are biochemical and it is important to stress the fact that before beginning this practice all students must take an intensive course on radioactivity handling and risks. Extreme caution should be used during the experiments so that students learn to work under safe conditions. Everything should be monitored after each assay in order to avoid contamination.

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