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PROTEIN KINASE IN *CICER* EMBRYONIC AXES

ALKA BANSAL, DAMAN SALUJA and R. C. SACHAR *

Biochemistry and Molecular Biology Laboratory, Department of Botany, University of Delhi, Delhi 110007, India

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Key Word Index—*Cicer arietinum*; Leguminosae; protein kinase; enzyme regulation; *de novo* biosynthesis; enzyme purification; subunit structure; characterization of reaction product.

Abstract—A dramatic increase in the cAMP-independent protein kinase activity (10-fold) was observed in the first 10 hr of germination of excised embryonic axes of *Cicer*. The lag phase of enzyme induction was very short since a 6-fold stimulation of protein kinase activity was witnessed after two hr of imbibition of the axes. The increase in protein kinase activity is ascribed to *de novo* biosynthesis of the enzyme. Conclusive proof for the *de novo* biosynthesis of protein kinase was obtained by labelling the proteins *in vivo* with [^{35}S]-sulphate and subsequently recovering the label predominantly in the methionine residues of the purified enzyme. The purification of the enzyme to electrophoretic homogeneity (453-fold) was achieved by ion exchange chromatography followed by affinity chromatography on a Casein-Sepharose column. Polyacrylamide gel electrophoresis of the [^{35}S]-labelled enzyme revealed a single radioactive band by autoradiography, that co-electrophoresed with the Coomassie Blue stained band of protein kinase. The M_r of the purified protein kinase is 94 000 as determined by molecular sieving on Sepharose CL-6B. SDS-PAGE data indicated that the enzyme is composed of two subunits of M_rs 49 000 and 62 000. Chemical characterization of the reaction product of protein kinase revealed that phosphorylation occurs at serine and threonine residues of the substrate, casein.

INTRODUCTION

Phosphorylation of polypeptides during post-translational modification plays an important role in the regulation of enzyme activity in eukaryotes [1]. Protein kinases are the key enzymes that are necessary for the phosphorylation of a variety of enzyme proteins [1]. Among plants, phosphorylation of proteins was shown by incubating the tissue in a medium containing [^{32}P]-orthophosphoric acid [2, 3]. Incubation of subcellular fractions with [γ-^{32}P]-ATP *in vitro* also resulted in the phosphorylation of several proteins [2, 4]. Both casein kinases and histone kinases have been isolated from plant extracts [5–8]. In soybean hypocotyls, an increase in the phosphorylation of several nuclear proteins has been reported in response to 2,4-D. This enhancement of phosphorylation of proteins was associated with the parallel rise in the nuclear protein kinase activity [9]. There has been no detailed study of the regulation of protein kinase at the onset of seed germination.

In this investigation, we report a significant enhancement of a cyclic nucleotide-independent protein kinase in the embryonic axes of *Cicer*. The *de novo* biosynthesis of protein kinase was unequivocally proved by labelling the enzyme *in vivo* during early germination of embryo. Protein kinase was purified to electrophoretic homogeneity and its M_r subunit structure and kinetic properties are discussed.

*Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

Induction of protein kinase in germinating excised embryonic axes of Cicer

Protein kinase activity was assayed in the G-25 fraction prepared from germinated *Cicer* embryonic axes. The substrate specificity of *Cicer* protein kinase was determined by testing the enzyme activity with casein, phosvitin, histones, protamine sulphate and bovine serum albumin. Casein proved to be the most effective phosphate acceptor (Table 1) and was therefore used as a substrate

Table 1. Substrate specificity of protein kinase in *Cicer* embryonic axes

Additions	Protein kinase activity (pmol Pi incorporated/ mg protein)	(Relative activity)
None	558	1.00
Casein	1401	2.5
Phosvitin	575	1.03
Histones	618	1.10
Bovine serum albumin	616	1.10
Protamine sulphate	529	0.94

Protein kinase was assayed in the G-25 fraction isolated from 48 hr germinated embryonic axes. Protein kinase was assayed in the presence of 500 μg each of casein, phosvitin, histones, protamine sulphate and bovine serum albumin.

Table 2. Summary of purification of protein kinase from *Cicer* embryonic axes

Step	Total protein (mg)	Sp. activity (pmol Pi incorporated/ mg protein)	Purification (Fold)	Yield (%)
Ammonium sulphate ppt (0–50% satn)	12140	277	1.00	100
DEAE-cellulose (0–0.2 M)	1596	930	3.36	45
Casein–Sepharose affinity chromatography	1.6	125000	453	6.1

Protein kinase activity was assayed at each step of enzyme purification using casein as substrate.

for the rest of the study. Protein kinase activity was linear up until 5 min of incubation at 30° and was insensitive to the addition of cyclic nucleotides (data not presented).

Early time-course studies of embryo germination indicated that enzyme induction occurs after an extremely short lag phase, since we could observe about a 6-fold stimulation of protein kinase after imbibing embryos for two hr (Fig. 1). The rapid rise in protein kinase activity during early germination of *Cicer* embryos could play a significant role in the efficient phosphorylation of a variety of enzyme proteins and thus serves as one of the major key enzymes in the post-translational regulation of gene expression.

Purification of protein kinase

Previous attempts to determine the M_r of the native protein kinase from wheat germ were unsuccessful [7, 10]. The inability to isolate protein kinase in its native form was ascribed to its denaturation or deaggregation during molecular sieving. In soybean cotyledons also, the M_r of the native enzyme is not known, although it has been stated that the fractionation of enzyme (CK II) on SDS-PAGE revealed three subunits [11]. In the present study, we have isolated a high M_r protein kinase from *Cicer* embryonic axes (Table 2). About a 453-fold purification of protein kinase was achieved by affinity chromatography on Casein-Sepharose column. After purification, the enzyme retained its activity for nearly 8 weeks at −20°. The purified protein kinase was electrophoretically homogeneous since it showed a single protein band on acrylamide gels (10%) (Fig. 2a). The M_r of the native enzyme is 94 000 as determined by molecular sieving on Sephadex G-150. SDS-PAGE of the purified enzyme revealed the heteromeric nature of the enzyme (Fig. 3). The M_rs of the two subunits were 62 000 and 49 000. The purified enzyme could phosphorylate casein and phosvitin, but did not accept histones as substrate (data not presented). The inability of the enzyme to phosphorylate histones could be due to the fact that it was purified by using a Casein-Sepharose affinity column. Phosphorylation of histones is apparently achieved by another class of kinases. The enzyme-catalysed reaction of purified protein kinase was linear for 30 min (data not presented). The enzyme exhibited a broad pH optimum (pH 7.0–8.5), with maximum activity at pH 8.0. The phosphorylation reaction was also dependent on Mg^{2+} ions. The maximum enzyme activity was obtained at 8 mM magnesium acetate. Chemical characterization of the reaction product of the enzyme showed that phosphorylation predominantly occurs at the threonine residues and to a lesser extent at the serine residues of the substrate protein (Fig. 4). Selective phosphorylation at threonine residues has also been reported in the chloroplast membrane polypeptides of pea [12]. In contrast, protein kinases ($ChlPK_1$ and $ChlPK_2$) isolated from spinach chloroplast phosphorylate only at the serine residues of the substrate casein [13]. In addition, tyrosine specific protein kinases have been reported in animal system [14]. Thus, it seems that different protein kinases could be selective in phosphorylating different amino acids of the common substrate.

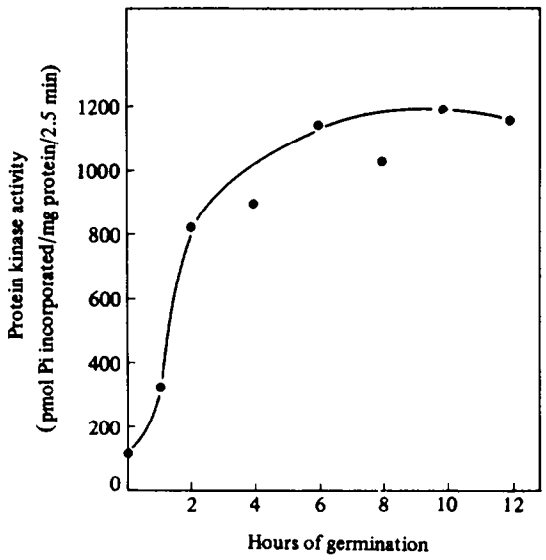

Fig. 1. Induction of protein kinase activity in excised embryonic axes of *Cicer*. Embryonic axes were scooped from 1 hr imbibed seeds of *Cicer* and cultured on a nutrient medium at 25° in the dark. Protein kinase was assayed in the G-25 fraction using casein as substrate.

De novo *biosynthesis of protein kinase during germination*

To establish whether the modulation of protein kinase activity in our system is achieved by *de novo* enzyme synthesis, or by the activation of pre-existing precursor

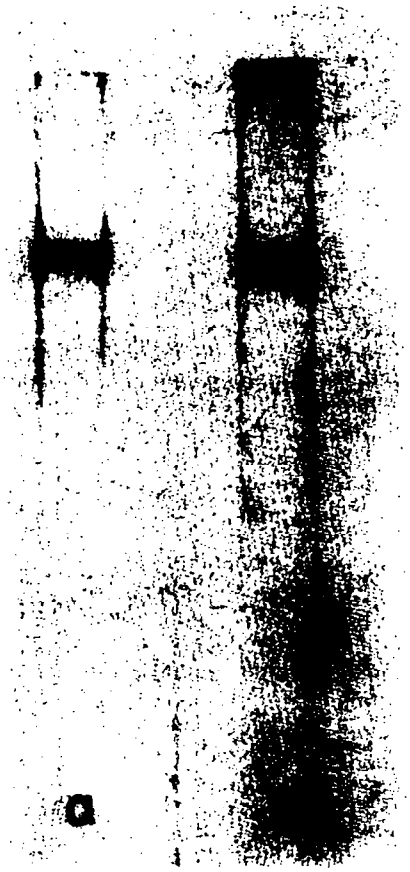

Fig. 2. Radioactive labelling of highly purified protein kinase with [^{35}S]-sulphate. (a) PAGE of the purified protein kinase on native gels (10%), showing a single protein stained band. (b) Autoradiograph of [^{35}S]-labelled protein kinase band on slab gels. Purified protein kinase isolated from labelled tissue was run on native gels (10%). Gels were stained, dried and autoradiographed.

enzyme molecules, we resorted to inhibitor approach and also attempted to label the enzyme *in vivo*. Cycloheximide (20 μg/ml) effectively inhibited (54%) protein kinase activity, thereby indicating the requirement of *de novo* protein synthesis for the enhancement of enzyme activity. A more direct proof for *de novo* enzyme biosynthesis was obtained by labelling the enzyme with [^{35}S]-sulphate under *in vivo* conditions. Polyacrylamide gel electrophoresis of the [^{35}S]-labelled purified enzyme followed by autoradiography of the dried gels, revealed a single radioactive band that co-electrophoresed with the protein kinase band stained with Coomassie Blue (Fig. 2b). Chemical characterization of the [^{35}S]-labelled protein kinase showed that the label could be recovered in the methionine and to a lesser extent in cysteine residues of the enzyme (Fig. 5). These studies thus unequivocally proved that the rise in the activity of protein kinase in germinating *Cicer* embryos is due to *de novo* biosynthesis of enzyme.

EXPERIMENTAL

Materials. The seeds of *Cicer arietinum* L. var. BG 257 were obtained from the Pulse Research Laboratory, Indian Agricultural Research Institute, New Delhi-12, India.

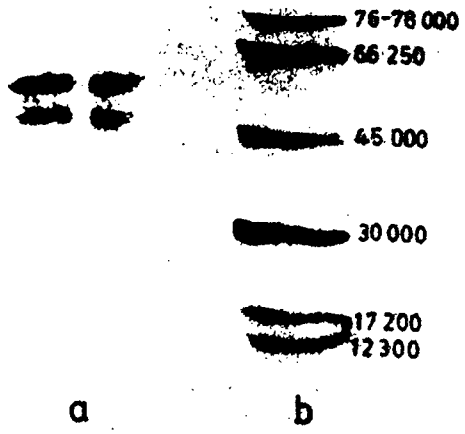

Fig. 3. SDS-PAGE of the purified protein kinase and SDS-protein markers. An aliquot of purified protein kinase was treated with SDS-buffer. The sample was subjected to electrophoresis on acrylamide gels (10%). Standard protein markers were also electrophoresed on the slab gel along with the sample protein. The gels were stained with Coomassie Blue. (a) Subunits of purified protein kinase. (b) Protein stained bands of standard protein markers.

Carrier free [^{32}P]-orthophosphoric acid and [^{35}S]-sulphuric acid were obtained from Bhabha Atomic Research Centre, Bombay, India. Sephadex G-150 and marker protein kit were from Pharmacia Fine Chemicals, Sweden. Phosphoamino acids (*O*-phospho-DL-serine, *O*-phospho-DL-threonine and *O*-phospho-DL-tyrosine) were from Sigma. Casein was dephosphorylated according to the procedure given in ref. [15]. [γ-^{32}P]-ATP was prepared enzymatically by the procedure of ref. [16].

Isolation and culture of excised embryonic axes. Cicer seeds were washed in running water for 2 hr. The embryonic axes were dissected manually and surface sterilized with $HgCl_2$ soln (0.02%) for 2 min. After a thorough rinse in sterile distilled water, the embryonic tissue was cultured on an asceptic liquid basal medium [17] in the dark at 25° for different intervals of time. Chloramphenicol (50 μg/ml) was added to the medium as a bactericidal agent.

Enzyme extraction. The germinated embryonic axes were homogenized in Tris–acetate buffer (25 mM, pH 7.6) containing β-mercaptoethanol (5 mM), magnesium acetate (3 mM) and polyvinyl-polypyrrolidone (PVP, 2% w/v). Acid washed sand was used as an abrasive for grinding the tissue. The homogenate was centrifuged at 20000 *g* for 15 min at 4°. The supernatant was fractionated by $(NH_4)_2SO_4$ (0–50% satn). The $(NH_4)_2SO_4$ fraction precipitate was desalted on a Sephadex G-25 column (1.8 cm × 10 cm). The preparation was designated as the G-25 fraction and was employed for the assay of protein kinase activity.

Protein estimation. Protein was estimated by the method described in ref. [18].

Assay of protein kinase activity. The standard protein kinase assay contained Tris–HCl buffer (20 mM, pH 8.0), magnesium acetate (18 mM), casein (500 μg), [γ-^{32}P]-ATP (30 nmol; 30 dpm/pmol) and the G-25 fraction (500 μg protein) in a final vol. of 500 μl. The assay was initiated by the addition of [γ-^{32}P]-ATP and incubated at 30° for 2.5 min. The reaction was terminated by adding an equal vol. of trichloroacetic acid (10%) containing sodium pyrophosphate (20 mM). Bovine serum albumin (500 μg) was also added as a carrier protein before

Fig. 4. Characterization of protein kinase reaction product as phosphoserine and phosphothreonine. The reaction product, [^{32}P]-labelled casein, was hydrolysed in 6 M HCl at 110° for 1 hr. Phosphoamino acids were separated by paper chromatography. The position of the [^{32}P]-serine and [^{32}P]-threonine was visualized by radioautography. The radioactive spots coincided with the authentic phosphoamino acids stained with ninhydrin.

terminating the reaction. The reaction mixture was then kept for 15 min at 80° and thereafter rapidly chilled for 30 min. The precipitate was collected on a Whatman 3 MM disc (25 mm) under suctional filtration. The precipitate was washed (× 6) with chilled TCA (5%, 40 ml), EtOH–Et_2O (1:1, 10 ml) and Et_2O (15 ml). The sample discs were dried at 60° for 1 hr and the radioactivity incorporated into the protein fraction was determined by liquid scintillation counting using a scintillation fluid containing toluene (1 l), PPO (5 g) and POPOP (0.3 g). Protein kinase activity was expressed as pmol phosphate incorporated per mg protein.

Purification of protein kinase. Extraction of protein kinase. Cicer embryonic axes were harvested from 48 hr germinated seeds and the tissue (800 g) homogenized in buffer A [Tris–acetate buffer (25 mM, pH 7.6) containing β-mercaptoethanol (5 mM), magnesium acetate (3 mM)]. Acid washed sand was used as an

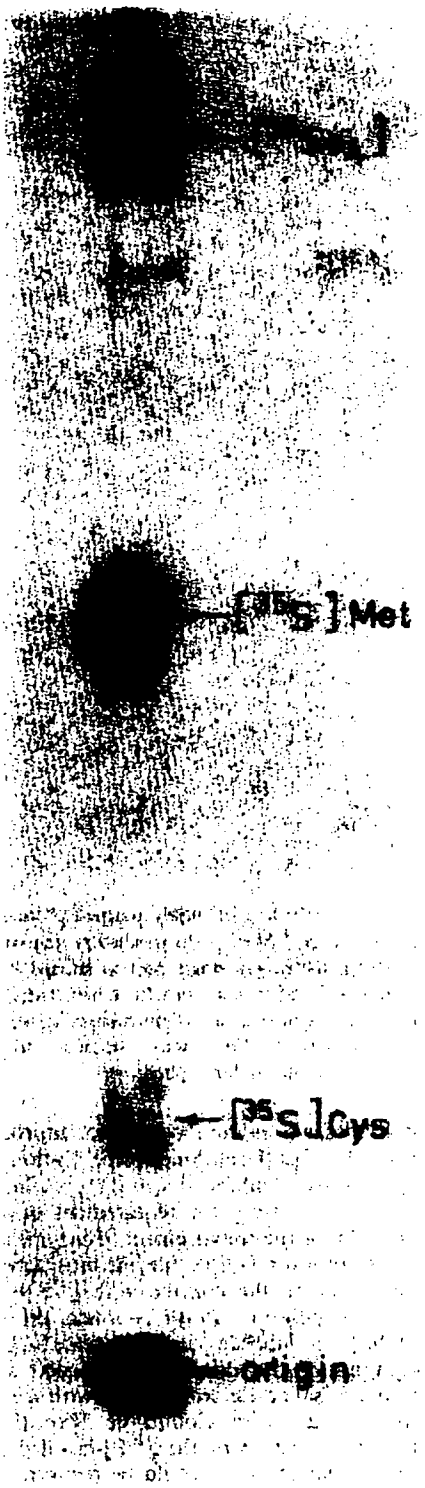

Fig. 5. Identification of [^{35}S]-labelled amino acids present in the *in vivo* labelled protein kinase. An aliquot of labelled, highly purified protein kinase was hydrolysed in 6 M HCl for 3 hr at 110°. Amino acids were separated by paper chromatography as described in methods. The dried chromatogram was exposed to Sakura X-ray film for 7 days at −20°. The autoradiograph revealed [^{35}S]-label in methionine and cysteine amino acids.

abrasive during homogenization. The homogenate was centrifuged at 20000 *g* for 15 min at 4° and the clear supernatant was subjected to $(NH_4)_2SO_4$ fractionation. The salt was removed from the $(NH_4)_2SO_4$ fraction precipitate (0–50% satn) by exhaustive dialysis against buffer A. All steps of enzyme purification were carried out at 4°.

DEAE-cellulose chromatography. The desalted $(NH_4)_2SO_4$ fraction (0–50%) was loaded at a concentration of 5 mg/ml on a column of DEAE-cellulose which had been equilibrated with buffer A. After binding the protein at a flow rate of 0.8 ml/min, the column was washed with two bed vol. of buffer A. Batch elution of the protein kinase major activity peak was achieved by eluting proteins with buffer A containing NaCl (0.2 M). The proteins in the eluant were precipitated with $(NH_4)_2SO_4$ (0–70% satn), suspended in buffer A and dialysed (2 l of buffer A × 2 changes) overnight. The desalted protein preparation was referred to as the DEAE-fraction.

Affinity chromatography. The DEAE-fraction was applied to a Casein-Sepharose column (1.5 cm × 10 cm) at a flow rate of 0.25 ml/min. The column was washed extensively with buffer A to remove unbound proteins. Protein kinase was subsequently eluted with buffer A containing KCl (0.5 M). Fractions containing protein kinase activity were pooled and concentrated with sucrose at 4°. The concentrated enzyme preparation was dialysed against buffer A to remove salt and excess of sucrose. This purified enzyme was stored in liquid N_2 and was used for the study of its kinetic properties. Protein was estimated according to the ref. [19] at all the steps of enzyme purification.

M_r *determination of purified protein kinase.* The M_r of protein kinase was determined by gel filtration. The purified enzyme (2 mg protein) was loaded on a Sephadex G-150 column (66 cm × 1.5 cm). Fractions of 3 ml each were collected after the void volume (V_0 = 58 ml). The protein content in each fraction was determined by absorbance measurements at 280 nm. The M_r of the purified enzyme was determined by calibrating the Sephadex G-150 column with standard protein markers of known M_r.

Treatment of embryos with cycloheximide. To study the effect of the translational inhibitor cycloheximide (CHI), the dry seeds were soaked in a soln of CHI (20 μg/ml) for 2 hr. Embryonic axes were excised manually and germinated for different intervals of time (0–48 hr) in the presence of CHI (20 μg/ml). Protein kinase activity was measured in the G-25 fraction.

In vivo *labelling of protein kinase to demonstrate* de novo *biosynthesis of protein kinase during early germination.* Embryonic axes were excised manually from 2 hr imbibed *Cicer* seeds. These were germinated on liquid basal medium in the presence of carrier free [^{35}S]-sulphate (2 mCi/ml). Protein kinase was purified to electrophoretic homogeneity by the above method and an aliquot of the preparation subjected to PAGE under non-denaturing conditions on 10% gels. The gels were stained and then dried using an LKB gel dryer. For the visualization of radioactive bands, the dried gels were exposed to Sakura X-ray films with intensifying screens at −20° for 6 days. The purified protein kinase was also subjected to acid hydrolysis for the identification of [^{35}S]-labelled amino acids. For this purpose, an aliquot of the labelled protein kinase was hydrolysed in HCl (6 M) for 3 hr at 110° under N_2 gas. The hydrolysate was chromatographed for 18 hr in a solvent system comprising of *n*-BuOH–HOAc–H_2O (12:3:5). The chromatogram was dried and kept for autoradiography for the detection of labelled amino acids. Authentic sulphur-containing amino acids (cysteine, methionine) were located by staining with ninhydrin.

PAGE. Acrylamide gels (10%) were prepared according to the ref. [20]. Purified enzyme up to 50 μg was run at a constant current (50 mA) in a LKB vertical gel electrophoresis unit. The gels were then stained with Coomassie Brilliant Blue (0.25%).

SDS-PAGE. The SDS-PAGE was carried out according to the method described in ref. [21].

Characterization of reaction product of protein kinase. The assay mixture (× 5) was incubated for 30 min at 30°. The reaction was terminated by TCA (10%) containing sodium pyrophosphate (20 mM). Carrier protein (BSA) was added at the time of terminating the reaction. The contents were heated at 80° for 15 min and subsequently chilled. The precipitate was collected by centrifugation at 17000 *g* for 10 min. The pellet was washed (× 4) with TCA (5%) containing sodium pyrophosphate (20 mM). The pellet was rinsed with EtOH–Et_2O (1:1) and air dried. The proteins were hydrolysed for 1 hr at 110° in HCl (6M) in an ampoule under N_2. The hydrolysate was subjected to PC for the separation of phosphoamino acids using two solvent systems: *n*-PrOH–HCl (0.5 N, 2:1) and *n*-BuOH–HOAc–H_2O (50:11:25). Authentic phosphoamino acids were detected with ninhydrin. The [^{32}P]-labelled spots were detected by autoradiography which was performed using intensifying screens and Sakura X-ray films. Exposure was at −20° for 4 days.

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