Pergamon

PII: S0031-9422(97)00054-X

Phytochemistry, Vol. 46, No. 3, pp. 461–467, 1997
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Printed in Great Britain
0031-9422/97 \$17.00+0.00

PURIFICATION AND CHARACTERISATION OF A PROTEIN KINASE FROM WINGED BEAN

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(*Received in revised form* 2 December 1996)

Key Word Index—*Psophocarpus tetragonolobus*; Fabaceae; winged bean; protein kinase; casein kinase I; enzyme purification.

Abstract—A soluble, cytoplasmic protein kinase was purified from the developing seeds of winged bean (*Psophocarpus tetragonolobus*) following conventional methods of protein purification including anion-exchange chromatography, gel-filtration and Blue Sepharose chromatography. The purified enzyme consists of a single polypeptide of M_r 45 000 as determined by SDS-PAGE and gel-filtration chromatography on Sephacryl S-200. The pH optimum of the protein kinase activity was 7.0, while the optimum concentration of Mg^{2+} was 5 mM. The enzyme utilised casein as an exogenous phosphate acceptor. The conventional modulators of protein kinases, including the cyclic nucleotides, Ca^{2+} and calmodulin, did not stimulate the purified enzyme. Heparin and spermine, too, had no effect on its activity. Phosphoamino acid analysis revealed that the enzyme transferred the γ-phosphate of ATP only to serine residues of casein. All these characteristics, taken together, classifies the purified protein kinase as a member of the casein kinase I group of enzymes. © 1997 Elsevier Science Ltd

INTRODUCTION

There has been growing interest in protein phosphorylation–dephosphorylation in plants during the past decade and a number of soluble as well as membrane-bound protein kinases have been identified, purified and characterised [1–6]. While the list of plant protein kinases is expanding rapidly, the role of protein phosphorylation in the regulation of cellular processes in plant systems still remains largely conjectural. It has, however, been shown to regulate, either directly or indirectly, the activity of several enzymes such as quinate:NAD oxidoreductase [7], ribulose bisphosphate carboxylase/oxygenase [8, 9], phosphoenol pyruvate carboxylase [10, 11], pyruvate phosphate dikinase [1], sucrose-phosphate synthase [12], pyruvate dehydrogenase complex [13, 14], and RNA polymerase II [6, 15]. The number of plant protein kinases studied to date, however, still remains very low compared with their numerous counterparts identified in mammalian cells alone during the last decade (see, for example [16–18] and refs therein).

The distribution of protein kinase activity in various tissues of winged bean [*Psophocarpus tetragonolobus* (L.) DC.], a leguminous crop from the tropics, has been examined earlier [19]. The changes in protein kinase activity and endogenous protein phosphorylation during the development of the seed and its subsequent germination have also been investigated. These studies indicated the presence of multiple protein kinases in the developing seeds, suggesting diverse roles for these enzymes in these tissues. To ascertain their specific physiological functions, it became necessary to identify each of these enzymes individually and characterise them. The present work describes an attempt to isolate and characterise one of these protein kinases from the developing seeds of winged bean.

RESULTS

Purification of protein kinase

Purification of the enzyme was performed five times; the data presented below describe a typical profile. All the following operations were conducted at 0–4°, except the enzyme assays which were performed at room temperature.

(1) *Preparation of tissue homogenate*

Developing (24–26 dpf) winged bean cotyledons (80 g), free from seed coats and embryonic axes, were

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Table 1. Summary of protein kinase purification from developing winged bean seeds

Steps of enzyme purification	Total protein (mg)	Specific activity (unit mg^{-1} protein)	Fold purification	Yield (%)
Tissue homogenate	1955	120	1	100
DEAE-cellulose peak I	1579	161	1.3	108.4
Sephacryl S-200 peak I	28	5630	46.9	67.2
Blue Sepharose	3	7530	62.7	9.6

The unit for enzyme activity is pmol ^{32}P incorporated min^{-1}. Protein estimations and enzyme assays were performed as described in the text. Casein was used as the substrate for the enzyme. Each assay was done in triplicate.

homogenised in 4 volumes of cold 25 mM Tris–HCl, pH 7.0, containing 4 mM $MgCl_2$, 1 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM benzamidine hydrochloride, 1 mM 2-mercaptoethanol, and a few drops of Antifoam C in a Sorvall Omni-mixer. Polyvinyl polypyrrolidone (PVPP) (1.5%) was then added and stirred for 5 min. The homogenate was centrifuged at 11 000 *g* for 20 min. The supernatant was filtered through cheese cloth and once again subjected to centrifugation at 100 000 *g* for 1 hr. The supernatant thus obtained has been referred to as the tissue homogenate. A total of 234 600 units of enzyme activity was present in this preparation with a specific activity of 120 units mg^{-1} protein (Table 1).

(2) *Anion-exchange chromatography*

The tissue homogenate was applied to a DEAE-cellulose column (2 × 24 cm), pre-equilibrated with 50 mM Tris–HCl, pH 7.0, containing 4 mM $MgCl_2$ (Buffer A). The column was then washed with 10 column volumes of this buffer. The bound proteins were eluted with 500 ml of a linear gradient of 0–0.5 M KCl, dissolved in the same buffer, at a flow-rate of 35 ml hr^{-1}. Two peaks of kinase activity, but only a single significant protein peak, could be resolved (Fig. 1, panel A).

(3) *Gel-filtration chromatography*

The DEAE-cellulose fractions (12–42), eluting with 0.15–0.3 M KCl and exhibiting relatively high enzyme activity, were pooled, concentrated and loaded onto a Sephacryl S-200 column (1.65 × 94 cm), pre-equilibrated with Buffer A. The flow-rate was maintained at 25 ml hr^{-1} and 3.35 ml fractions were collected. Three peaks of protein kinase activity could be resolved (Fig. 1, panel B).

(4) *Blue Sepharose-affinity chromatography*

Gel-filtration fractions 34–38, comprising the second activity peak, were pooled, concentrated and applied to a Blue Sepharose column (1 × 4 cm), again pre-equilibrated with Buffer A. The column was washed with 10 column volumes of the same buffer, followed by the elution of the bound proteins with 50 ml of a linear gradient of 0–0.6 M KCl, dissolved in Buffer A. The activity eluted as a single peak between 0.3 and 0.4 M KCl, but did not coincide with the single protein peak obtained (Fig. 1, panel C).

The purification procedure resulted in the recovering of 9.6% of the original enzyme activity, with about 63-fold purification (Table 1).

Homogeneity and M_r

The protein kinase preparation, obtained after Blue Sepharose-affinity chromatography, showed one major silver-stained band on SDS-PAGE analysis, although a few minor bands could also be seen (Fig. 2). All subsequent characterisation of the enzyme was carried out with this purified preparation.

The M_r of the protein kinase was shown to be about 45 000 by gel-filtration on Sephacryl S-200 (Fig. 3). Since SDS-PAGE analysis of this enzyme revealed a single band corresponding to the same M_r, the protein kinase consisted of a single polypeptide.

Optimum pH for the kinase activity

The enzyme, when assayed at different pH in the absence of any exogenous substrate, showed maximum activity at pH 7.0 (Fig. 4). Each assay was carried out for 1 min during which period the enzyme activity was observed to be linear. All enzyme assays were subsequently performed at the optimum pH of 7.0.

Substrate specificity

The purified enzyme was assayed with a number of different exogenous substrates typical of protein kinases. Of these, only casein was significantly phosphorylated by the enzyme, with an approximate sevenfold increase in its activity over that of the control without any exogenous substrate. Histone and protamin led to an insignificant increase of 1.3- and 1.2-fold, respectively, while phosvitin showed no elevation in activity at all (Fig. 5). These results showed that the

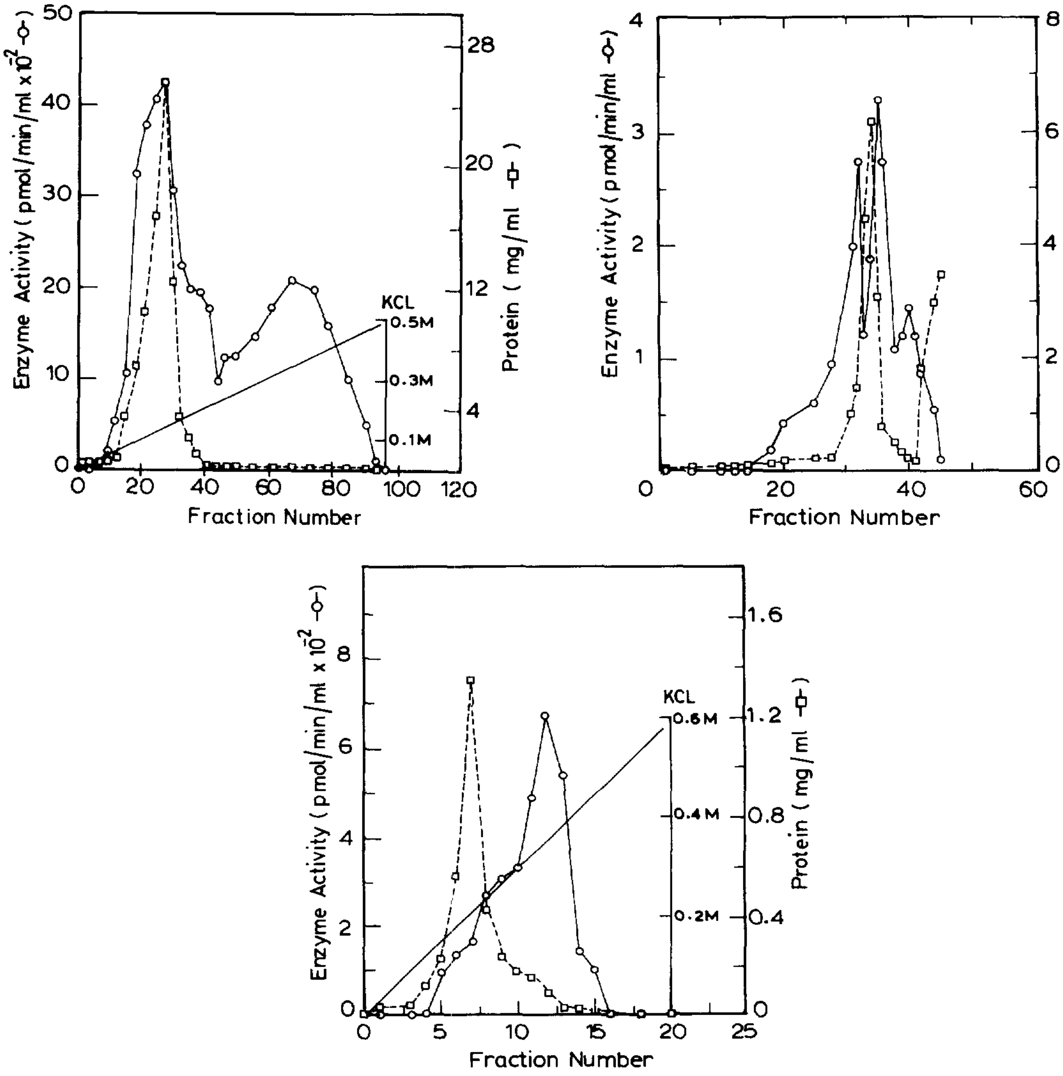

Fig. 1. Elution profile of the winged bean soluble protein kinase activity and total protein at successive stages of purification. (A) DEAE-cellulose chromatography of the seed extract; (B) Gel-filtration chromatography of DEAE-cellulose peak I; and (C) Blue Sepharose chromatography of gel-filtration peak II. Protein estimations and kinase assays (using casein as the exogenous substrate) were performed as described in the text. The continuous straight line in panels A and C represents the concentration gradient of KCl.

purified protein is a putative member of the casein kinase family of protein kinases.

The enzyme failed to phosphorylate casein when GTP was used as a phosphate donor instead of ATP (data not shown). This clearly indicated that it belongs to the casein kinase I group of enzymes.

Effect of cations

There was an absolute requirement of Mg^{2+} for the protein kinase activity, the optimum concentration being 5 mM (Table 2). Other cations such as Ca^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, or NH_4^+ could not substitute for Mg^{2+}. In the presence of Mg^{2+}, Ca^{2+} did not affect enzyme activity, although Mn^{2+}, Zn^{2+} and Hg^{2+} and NH_4^+ showed different levels of inhibition (Fig. 6).

Effect of calmodulin and cyclic nucleotides

Ca^{2+}-calmodulin and cyclic nucleotides like cAMP and cGMP are known to regulate a number of different protein kinases. Calmodulin, however, had no effect on the activity of the purified enzyme, either in the presence or in the absence of calcium (data not shown). The cyclic nucleotides cAMP and cGMP also did not significantly affect the protein kinase activity (data not shown).

Effect of thiol reagents

The activity of the purified protein kinase was not affected by DTT or 2-mercaptoethanol (data not shown). Thiol-blocking reagents like *p*-chloro-

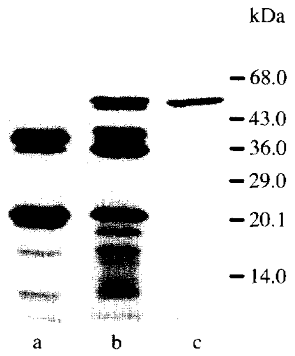

Fig. 2. SDS-PAGE analysis of protein at different stages of purification of winged bean soluble protein kinase. Proteins from the pooled fractions of each step of purification were resolved on a 12% denaturing polyacrylamide gel. Lane a, DEAE cellulose peak I; lane b, gel filtration peak II; lane c, Blue Sepharose eluted fractions. Equal amounts of proteins were applied to each lane and the bands were visualised by silver staining.

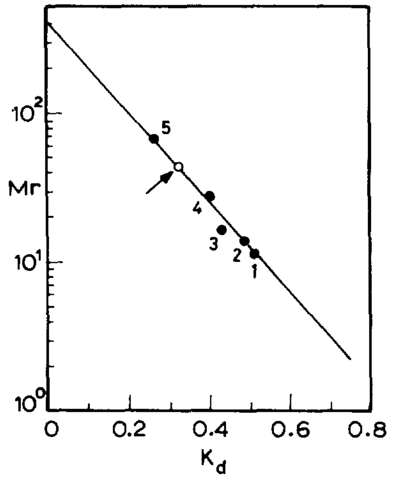

Fig. 3. Determination of M_r of the winged bean soluble protein kinase by gel-filtration chromatography on Sephacryl S-200 column. Blue dextran and bromophenol blue were used to determine the void volume and the internal volume, respectively. The marker proteins used were: 1, cytochrome C (12 500); 2, lysozyme (14 300); 3, myoglobin (17 200); 4, carbonic anhydrase (29 000); 5, bovine serum albumin (68 000). The arrow indicates the position of the purified protein kinase.

mercuribenzoate (*p*-CMB) or 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) also did not affect the enzyme activity (data not shown). Heparin or spermine were also unable to significantly modulate the activity of the purified protein kinase (data not shown).

Phosphoamino acid analysis of phosphorylated casein

Casein was phosphorylated by the purified kinase in the presence of [γ-^{32}P]ATP *in vitro*, and the reaction product hydrolysed to release the phosphoamino acids. Analysis of the hydrolysate by paper chro-

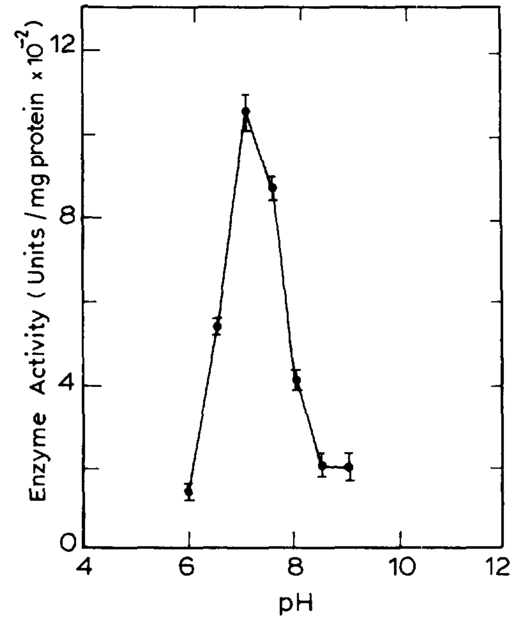

Fig. 4. Activity of winged bean soluble protein kinase at different pH. The assays were carried out for 1 min in the absence of any exogenous substrate. Buffers used were MES (pH 6.0), PIPES (pH 6.5) and Tris–chloride (pH 7.0–9.0). The experiment was repeated three times, each time in triplicate. The bars at each point represent standard errors of the means.

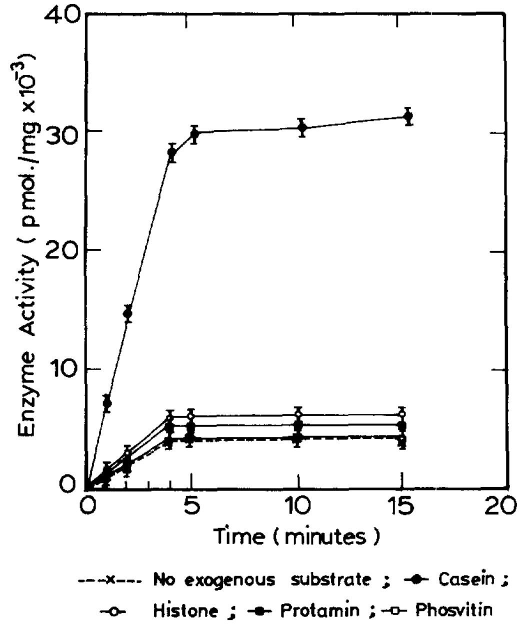

Fig. 5. Substrate specificity of the purified winged bean soluble protein kinase. The enzyme was assayed as described in the text for different periods of time, in the presence or absence of exogenous substrates. The experiment was repeated three times, each in triplicate. The bars at each point represent standard errors of the means.

Table 2. Magnesium ion requirement for the activity of winged bean seed protein kinase

Magnesium (mM)	Enzyme activity (unit mg^{-1} protein)
—	78±15
0.5	2400±96
1.0	3050±102
2.0	4150±98
3.0	4950±110
4.0	5790±109
5.0	7180±122
6.0	6030±112
7.0	5850±115
8.0	5550±100
9.0	5200±105
10.0	5200±95

The unit for enzyme activity is pmol ^{32}P incorporated min^{-1}. Values are means ± SE of three experiments, each performed in triplicate. Protein kinase assays were carried out as described in the text. Casein was used as the exogenous substrate for the enzyme.

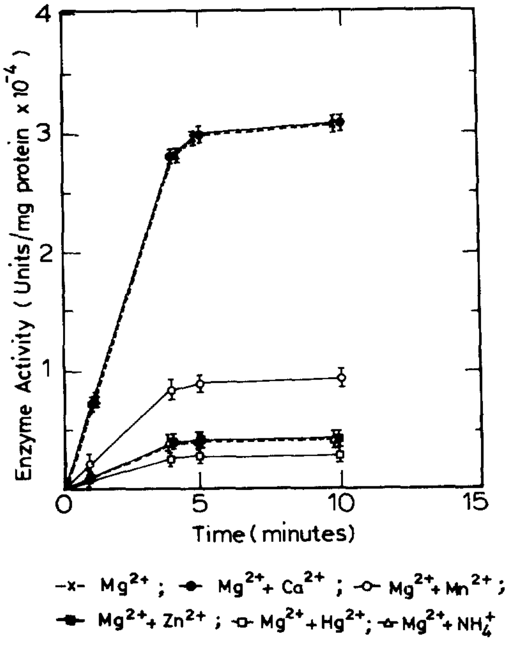

Fig. 6. Effect of various cations on the activity of the soluble protein kinase from winged bean seeds. The enzyme was assayed as described in the text for different periods of time. Casein served as the exogenous substrate in all assays. The experiment was repeated three times, each time in triplicate. The bars at each point represent standard errors of the means.

matography followed by autoradiography, showed a single radioactive spot that matched with that of the standard phosphoserine (Fig. 7). Thus, the purified protein kinase belongs to the category of serine-specific kinases.

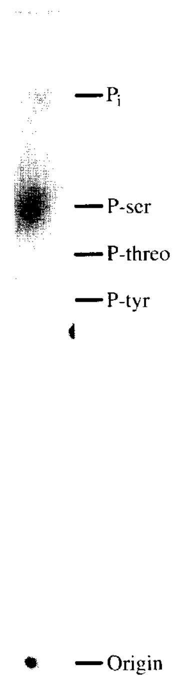

Fig. 7. Identification of the amino acid moiety on casein phosphorylated by the purified winged bean soluble protein kinase. The ^{32}P-labelled product was hydrolysed in 6 N HCl and the hydrolysate was analysed by cellulose thin-layer electrophoresis as described in the text. The labelled spots were detected by autoradiography. The positions of the standards (P-ser, phosphoserine; P-threo, phosphothreonine and P-tyr, phosphotyrosine), visualised by ninhydrin staining, have been indicated on the right-hand margin.

DISCUSSION

This investigation reports the purification and characterisation of a soluble protein kinase from the developing seeds of winged bean (*Psophocarpus tetragonolobus*). The enzyme has been classified as a casein kinase not only on the basis of its relatively higher affinity for casein compared with other substrates, but also because it lacks some of the usual modes of regulation such as that by cyclic nucleotides Ca^{2+} and calmodulin. Following convention as applied to animal casein kinases [20], the first peak of the DEAE-cellulose-eluted enzyme has been called casein kinase I (CK-I). Some of the physico-chemical properties also suggest that the purified protein belongs to the casein kinase I group of enzymes. Like the other members of the group (and unlike the casein kinase II group of enzymes), this protein is monomeric in nature, has a relatively low M_r (45 000—as determined by gel filtration and SDS-PAGE), and is neither inhibited by heparin nor stimulated by spermine. Moreover, it can only phosphorylate serine, but not threonine, residues of casein. The enzyme is active only between pH 7.0 and 8.0 and, therefore, differs from the casein kinase II enzymes which are active over a much wider range of pH. The casein kinases I and II also differ in their choice of phosphate donor. While the former can utilise only ATP, the latter is capable of using both ATP and GTP. The purified enzyme can only use ATP as the donor of the phosphoryl group. Taken together, these characteristics of the winged bean seed enzyme allow it to be placed quite reasonably in the casein kinase I group of enzymes [21].

The inhibition of the kinase activity by Hg^{2+} indicates the likely involvement of free sulphydryl groups in the catalytic process. Thiol-blocking reagents, like *p*-CMB and DTNB, in contrast do not have any effect on the enzyme activity. This could be possibly attributed to the inaccessibility of these reagents to the cysteine residues in the active site of the enzyme due to steric hindrance.

The purification of protein kinases is a difficult task owing to the presence of a number of different types of the enzyme, with similar properties, in tissues at particular stages of development and differentiation. Moreover, the presence of endogenous substrates, together with the enzymes in the same fraction, results in the formation of reasonably stable enzyme–substrate complexes. These are liable to behave differently from the free enzymes, and therefore, can give a false impression of a new enzyme altogether. To avoid this confusion, excess of exogenous substrate (i.e. casein) has been added to select out the casein kinases by outcompeting the other endogenous substrates. Assay of the crude tissue homogenate was difficult due to the presence of interfering enzymes like phosphatases and ATPases. It was, therefore, not possible to make an accurate quantitative assessment of the enzyme at this stage of purification.

The elution profile of the enzyme after DEAE-cellulose chromatography consisted of two activity peaks; both could utilise casein as exogenous substrate. This is a very common feature of animal protein kinases [22], and has also been reported for the soybean enzymes [23]. A close examination of the results of anion-exchange chromatography made it obvious that there were more than one type of protein kinase in each of the activity peaks since their elution profiles did not follow Gaussian distribution patterns. This conclusion was further strengthened by the subsequent resolution of the DEAE-cellulose peak I into three distinct enzymatically active fractions following gel-filtration. This, therefore, quite clearly established the existence of multiple protein kinases in the developing winged bean seeds. This conclusion is also consistent with the earlier discovery of a number of autophosphorylated protein kinases in these tissues [19].

A number of casein kinases have been purified from plants [23–25]. Of these, only those isolated from soybean cotelydons [23] are similar to the winged bean enzyme. Interestingly, both these plant species belong to the Fabaceae.

A serine protease has recently been isolated from the developing seeds of winged bean (Usha, R. and Singh, M., pers. commun.). Although present in reasonable amounts, this enzyme exhibits relatively low activity. Since the purified protein kinase described here is capable of phosphorylating serine residues, it could function in the temporal regulation of such a protease, through phosphorylation of its active site serine residues. Nevertheless, a distinct physiological role for the protein kinase can only be ascertained after the discovery of its natural substrate(s).

EXPERIMENTAL

Plant materials. Seeds of a local variety of winged bean, *Psophocarpus tetragonolobus* (L.) DC., were collected from the garden of the Indian Institute of Chemical Biology, Calcutta. The stages of seed development were determined by tagging the pods immediately after their appearance, taken to be the first day after pod formation (dpf).

Chemicals. Blue Sepharose, Sephadex G-50 (medium), and Sephacryl S-200 were obtained from Pharmacia. Coomassie Brilliant Blue R-250 or G-250 and DEAE-cellulose were purchase from Pierce, while benzamidine hydrochloride was from Aldrich. Acrylamide, *N*,*N*-methylene bis acrylamide and CCl_3COOH (TCA) were obtained from Sisco Research Laboratories, India, and Cocktail 'O'-scintillation fluid from Spectrochem, India. Radiochemicals including [γ-^{32}P]ATP (3000 Ci/m mol) and carrier-free [^{32}P] were supplied by Bhabha Atomic Research Centre, Bombay, India. All other chemicals were obtained from Sigma.

Protein kinase assay. Protein kinase activity was assayed as follows. The reaction mixt. contained 50 mM Tris–HCl, pH 7.0, 5 mM $MgCl_2$, 1 mM EDTA, 25 μg bovine serum albumin (BSA), 2 mM NaF, 50 μM Na vanadate, 7 mg ml^{-1} *p*-nitrophenyl phosphate (PNPP), 20 μg dephosphorylated casein (unless otherwise stated) and 100 μM [γ-^{32}P]ATP (sp. act. of 100–3000 cpm pmol^{-1}, as required) in a total vol. of 50 μl. The reaction was initiated by the addition of the tissue extract or enzyme prepn. After incubation for a specified period of time at room temp. (27°), 25 μl aliquots of the reaction mixt. were spotted on Whatman 3 MM paper discs, presoaked in cold 25% TCA containing 20 mM PPi and 10 mM adenosine. The paper discs were extensively washed with 5% TCA containing 20 mM PPi, and total radioactivity from protein-bound ^{32}P determined in a LKB Rackbeta liquid scintillation counter. All assays were done in triplicates. The assay was also carried out once using [γ-^{32}P]GTP instead of [γ-^{32}P]ATP. Finally, it may be noted that BSA, used in the assay mixture, did not act as a substrate for the enzyme (data not shown). One enzyme unit has been defined as the amount of enzyme that catalyses the transfer of 1 pmol of phosphate per min, under the conditions of the assay.

Protein determination. Protein was determined by the method of ref. [26] using BSA as a standard.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed in 12% gels according to ref. [27]. Protein bands were visualised by the Ag-staining method [28].

M_r *determination.* The purified protein kinase was subjected to gel-filtration on a Sephacryl S-200 (1.65 × 94 cm) column. The flow-rate through the column was maintained at 25 ml hr^{-1} and 3.35 ml frs were collected. Blue dextran and bromophenol blue were used to determine the void and internal vols of the column, respectively. The M_r markers used were cytochrome C (M_r 12 500), lysozyme (M_r 14 300), myoglobin (M_r 17 200), carbonic anhydrase (M_r 29 000) and bovine serum albumin (M_r 68 000).

Phosphoamino acid analysis. Casein was phosphorylated by the purified enzyme as described in the assay, except that the total reaction mixt. was 100 μl and the time of incubation was 1 hr. The ^{32}P-labelled product was precipitated with 10% TCA containing 20 mM PPi. The ppt was washed × 3 with Me_2CO, dried and hydrolysed in 0.5 ml 6 M HCl at 100° for 6 hr. The hydrolysate was air-dried and dissolved in 0.5 ml of a mixt. of $HCOOH–HOAc–H_2O$ (1:3:36), pH 1.9. A 10 μl sample was then applied to the cathode edge of a cellulose thin-layer sheet (Eastman 13250) and electrophoresed at 450 V for 90 min in a pH 3.5 buffer system (HOAc–pyridine–H_2O, 10:1:189) [29]. Phosphoserine, phosphothreonine and phosphotyrosine were used as standards. The cellulose sheets were dried and radioactive spots located by autoradiography, while the standards were visualised with ninhydrin.

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