Pergamon

PII: S0031-9422(97)00053-8

Phytochemistry, Vol. 46, No. 3, pp. 455–459, 1997

Printed in Great Britain
0031-9422/97 $17.00+0.00

ENDOGENOUS PROTEIN PHOSPHORYLATION AND PROTEIN KINASE ACTIVITY IN WINGED BEAN

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(*Received in revised form* 2 *December* 1996)

Key Word Index—*Psophocarpus tetragonolobus*; Fabaceae; winged bean; protein kinase; protein phosphorylation; seed development; seed germination.

Abstract—In winged bean (*Psophocarpus tetragonolobus*) protein kinases (E.C. 2.7.1.37) were found in all tissues studied. There was a significant increase in kinase activity during seed development, with a concomitant enhancement in the phosphorylation of a number of polypeptides; this was reversed in germinating seed cotyledons. Protein phosphorylation was apparently correlated with the increase in the protein content of the developing seed and the growing axis. At least three distinct autophosphorylating proteins could be distinguished in the developing seeds after SDS-PAGE, indicating the presence of different types of protein kinases in winged bean. ©1997 Elsevier Science Ltd

INTRODUCTION

Protein kinases are ubiquitous in eukaryotic cells and post-translational phosphorylation and dephosphorylation play vital regulatory roles in many metabolic processes [1–4]. In animal cells, study of a number of protein phosphorylation–dephosphorylation cascades has contributed to our understanding of the mode of action of hormones and the regulation of a number of key metabolic enzymes at the molecular level [5, 6]. In addition, many other cellular processes, such as cell division and differentiation and signal transduction, are regulated by protein phosphorylation and dephosphorylation [3, 7–10].

During seed development, rapid cell division occurs initially, and is followed by the differentiation of these cells into the embryonic axis and cotyledon(s). There is a subsequent period of cell expansion and elongation which is marked by high rates of protein synthesis. This results in a large accumulation of proteins, principally storage proteins, in the seed. Although there exists some idea about the involvement of these proteins and their regulators in seed development [11, 12], very little is known about the role of protein phosphorylation–dephosphorylation in these processes.

Winged bean, *Psophocarpus tetragonolobus* (L.) DC., a widely cultivated tropical leguminous plant, has received great attention due to its high nutritional value [13]. Extensive work has been conducted on proteases and their inhibitors [14, 15], lectins [16] and storage proteins [17] in the seeds of this plant. However, nothing is known about the mechanisms regulating the various metabolic processes. The distribution of protein kinase activity in various tissues of this plant has now been examined, and the changes in protein kinase activity and endogenous protein phosphorylation during the development of the seed and its subsequent germination have been investigated.

RESULTS AND DISCUSSION

Distribution of protein kinases in winged bean tissues

Protein kinase activity was detected in all the tissues of winged bean that were examined. This activity is constituted by a group of kinases which use endogenous proteins and/or casein as their substrates. Table 1 presents both the total and the specific activity of these enzymes in various tissues. The developing seed cotyledons have the highest amounts of total enzyme activity followed by tuber, leaf, epicotyl, germinating seed cotyledon and root, in that order. The germinating seeds, on the other hand, have the highest protein content resulting in the lowest specific activity of the kinases in this tissue.

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Table 1. Distribution of protein kinase activity in different winged bean tissues

Tissue	Total activity (unit g^{-1} FW)	Specific activity (unit mg^{-1} protein)
Seed cotyledon:		
Developing seed (10 dpf)	1351 ± 126	102 ± 19
Developing seed (26 dpf)	2905 ± 137	136 ± 39
Germinating seed (1 day)	514 ± 36	10 ± 2
Tuber	1150 ± 110	121 ± 17
Leaf	855 ± 52	94 ± 10
Epicotyl	591 ± 47	258 ± 27
Root	462 ± 54	249 ± 20

A unit of enzyme activity has been expressed in terms of pmol ^{32}P incorporated min^{-1}. Values shown are means ± s.e. for three experiments, each performed in triplicate. Tissue extracts were assayed for protein kinase activity as described in the text. Casein was used as the exogenous substrate for the enzyme. dpf, days after pod formation; FW, fresh weight.

Developing seeds had a considerably higher level of protein kinase activity in comparison with the other tissues examined. Since such seeds are known to be sites of intense metabolic activity, particularly during embryogenesis, these were chosen for further study.

Changes in protein kinase activity and total protein during the development of winged bean seeds

The patterns of protein kinase activity and the total protein content in developing winged bean seeds are shown in Fig. 1. The total kinase activity was considerable, even as early as 10 days after pod formation (dpf), increased further with development to reach a maximum level at around 26 dpf, and thereafter fell sharply. The activity profile was found to be completely different from that of the total soluble protein as the latter increased very slowly during the early stages of development with a substantial rise only around 30 dpf (Fig. 1). After that it reached a plateau and there was no further change even at the desiccation stage.

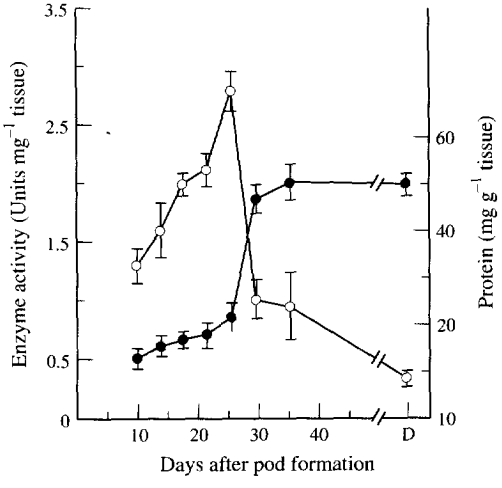

Fig. 1. Protein kinase activity and total protein content of developing winged bean seeds. Total kinase activity (open circle) was assayed and total protein content (closed circle) estimated as described in the text. Casein was used as the exogenous substrate in the assay. Each point represents the mean of three independent assays done in triplicate; the bars represent standard deviations of the means. D—mature desiccated stage.

In winged bean, storage proteins are synthesised and accumulated during the later stages of seed maturation, commencing around 25 dpf [15]. This is followed by a period when major metabolic activities are gradually suspended and desiccation is triggered (29 dpf onwards). The synthesis of storage proteins also cease at this stage. The sharp fall of protein kinase activity coincided with the onset of the late maturation phase, and gradually decreased thereafter with increasing desiccation of the seed.

Changes in the phosphorylation of endogenous proteins during seed development

The changes in the profile of endogenous proteins in the developing seeds phosphorylated *in vitro* are shown in Fig. 2. Two major phosphorylated protein

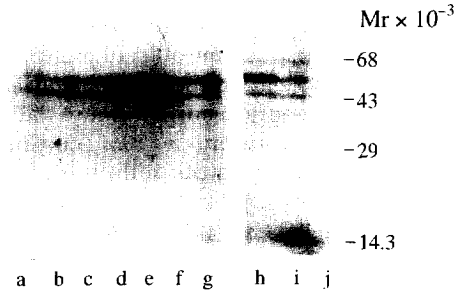

Fig. 2. Phosphorylation of endogenous proteins during the development of winged bean seeds. The phosphorylated products were analysed by SDS-PAGE and autoradiography as described in the text. Developmental stages in dpf: Lanes a—10, b—13, c—16, d—19, e—22, f—24, g—26, h—26, i—30 and j—36. M_r markers are shown on the right margin.

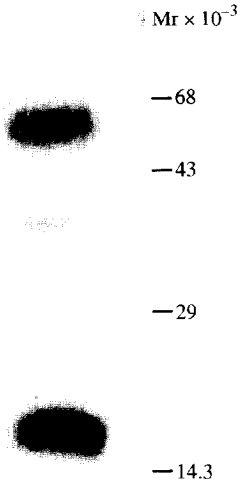

Fig. 3. Autophosphorylating proteins in developing winged bean seeds (26 dpf). The seed extracts were subjected to SDS-PAGE (12%); the separated proteins were then denatured, renatured and subsequently autophosphorylated as described in the text. M_r markers are shown on the right margin.

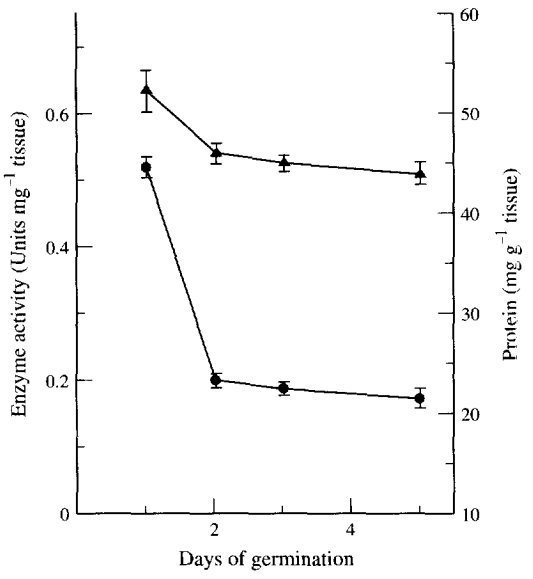

Fig. 4. Protein kinase activity and total protein content of germinating winged bean seeds. Kinase activity (closed circle) and protein content (closed triangle) were measured as described in the text with casein as the exogenous substrate in the enzyme assay.

bands (55 and 48 kDa) and some minor bands were observed at all stages of development. The intensity of the bands increased gradually from 10 dpf to 26 dpf (Fig. 2, lanes a–h), and decreased thereafter (Fig. 2, lanes i and j). This pattern of phosphorylation conformed to the observed rise and fall in the protein kinase activity of the seeds, discussed above.

Autophosphorylated protein kinases in the developing seeds

Phosphorylation of proteins *in situ*, following SDS-PAGE, has been employed in the analysis of purified protein kinases from animals [18, 19], yeast [20] and plants [21–23], as well as in tissue homogenates [24]. Using this approach, three distinct autophosphorylated protein bands were identified in developing seeds (26 dpf) (Fig. 3) and in the embryonic axes (data not shown). The mobilities of these bands indicated apparent M_rs of 46, 34 and 15 k, respectively. This possibly indicated a multiplicity of protein kinases in growing tissues. No radioactive bands were, however, visible when the gel was incubated with [α-^{32}P]ATP; this ruled out the possibility of these being other ATP-binding proteins (data not shown).

Protein kinase activity and endogenous protein phosphorylation in the germinating cotyledons

During early stages of germination there was only a slow decline in protein content, whereas protein kinase activity decreased sharply on the second day of germination and remained at this lower level without further significant change (Fig. 4). The reduced protein kinase activity in the cotyledons, as germination progressed, was also reflected in the almost undetectable levels of phosphorylated proteins from the second day of germination onwards (data not shown).

Protein kinase activity and endogenous protein phosphorylation in the growing embryonic axes

Total protein kinase activity and the protein content of the embryonic axes at different stages of germination are shown in Fig. 5. There was a steady

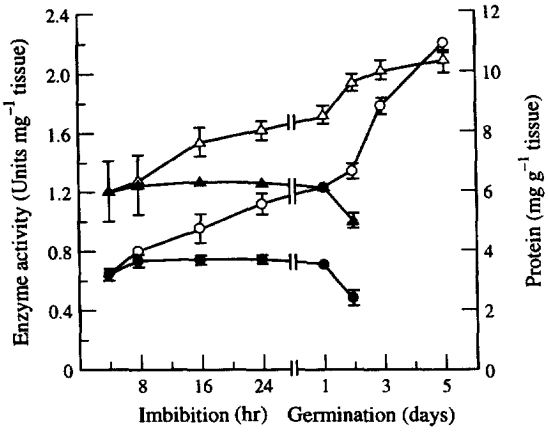

Fig. 5. Protein kinase activity and total protein content of the embryonic axes of germinating winged bean seeds: general profile and the effect of cycloheximide. For cycloheximide treatment, seeds were imbibed and germinated in the presence of 10 μg ml^{-1} of the chemical. Kinase activity (untreated ○; cycloheximide-treated ●) and protein content (untreated △; treated ▲) were measured as described in the text with casein as the exogenous substrate in the assay.

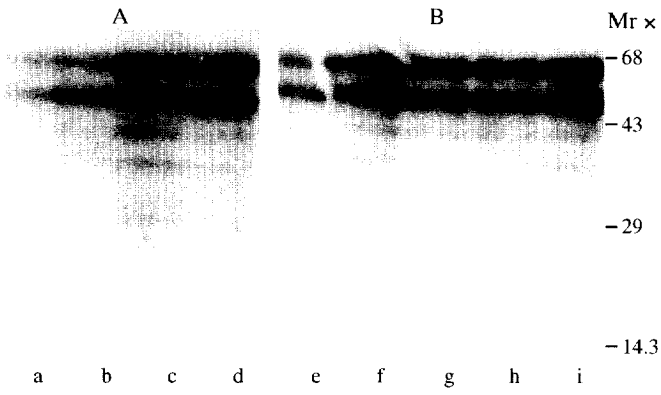

Fig. 6. Phosphorylation of endogenous proteins in the embryonic axes of germinating winged bean seeds. The phosphorylated products were analysed by SDS-PAGE and autoradiography as described in the text. A: hours of imbibition: Lanes a—4, b—10, c—16 and d—24. B: Days of germination: Lanes e—1, f—2, g—3, h—5 and i—7. *M*, markers are shown on the right margin.

increase in kinase activity from 4 hr of imbibition to the fifth day of germination. The total protein content also increased gradually soon after the commencement of imbibition. Cycloheximide treatment of the seeds at the time of imbibition completely abolished the increase of the enzyme activity as well as the total protein content; this was a clear indication that the increase of kinase activity involved the *de novo* synthesis of either the enzyme(s) or that of any protein influencing its activity (Fig. 5).

The results of the analysis of the phosphorylated endogenous proteins in the growing embryo are presented in Fig. 6. There was a steady increase in the intensity of two major protein bands (55 and 48 kDa) as well as that of some minor ones with the progress of imbibition [Fig. 6 (A)] and germination [Fig. 6 (B)].

Intense metabolic activities occur during embryogenesis and seed development. After attaining maturity, seeds undergo desiccation and enter dormancy when all metabolic activities cease. The subsequent onset of germination triggers the resumption of anabolic activities in the growing embryo, while the cotyledons of the germinating seeds undergo a dramatic reversal of activities leading to the mobilisation and degradation of reserve storage materials. The association of protein kinases with these tissues showing active growth and metabolism, suggests the possible involvement of protein phosphorylation in different regulatory processes at various phases of seed development and embryo growth.

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).

Germination of winged bean seeds. Sterilised winged bean seeds were imbibed for 24 hr and then germinated on moist blotting paper wrapped around glass plates at 27° [25]. Seeds imbibed for 24 hr were designated to be on day 0 of germination. The seed coats were observed to crack the next day, termed as the first day of germination.

Preparation of tissue extract. Cotyledons, free from seed coats, were used for making extracts from developing seeds. While preparing extracts from germinating seeds, the cotyledons without the seed coats and the embryonic axes were used separately.

All plant tissues were homogenised in 5 vol. 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 1.5% polyvinyl polypyrrolidone at 4° in a glass hand-held homogeniser. The homogenates were centrifuged at 11 000 *g* for 20 min at 4° and the supernatant used for biochemical assays.

Protein kinase assay. Protein kinases were assayed as follows. The reaction mixture contained 50 mM Tris–HCl, pH 7.0, 10 mM $MgCl_2$, 1 mM EDTA, 25 μg bovine serum albumin, 2 mM NaF, 50 μM Na vanadate, 7 mg ml^{-1} paranitrophenyl phosphate (PNPP), 20 μg dephosphorylated casein (as the exogenous substrate) (Sigma) and 100 μM [γ-^{32}P]ATP (specific activity of 1.85–55.50 Bq $pmol^{-1}$, as required) in a total vol. of 50 μl. The reaction was initiated by the addition of the tissue extract. After incubation for a maximum time of 15 sec (see below) at room temp. (27°), 25 μl aliquots of the reaction mixt. were spotted on Whatman 3 MM paper discs, presoaked in cold 25% CCl_3COOH (TCA) containing 20 mM PP_i and 10 mM adenosine. The paper discs were extensively washed with 5% TCA containing 20 mM PP_i, and total radioactivity from protein-bound ^{32}P determined in a LKB Rackbeta liquid scintillation counter. All assays were done in triplicates.

Total protein kinase activity in extracts of winged bean seeds, assayed as described above, was observed to be initially linear only for about 15 sec. Therefore,

for all tissues, the assays were performed for that length of time and the enzymatic rates calcd from these velocities.

One enzyme unit is defined as the amount of enzyme that catalyses the transfer of 1 pmol of phosphate per min, under the conditions of the assay.

Electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% gels according to ref. [26].

Analysis of phosphorylated endogenous proteins by SDS-PAGE and autoradiography. Enzyme extracts (containing equal quantities of protein) were incubated with 50 μM [γ-^{32}P]ATP (sp. act. of 92.5 Bq pmol^{-1}), 10 mM $MgCl_2$, 2 mM NaF, 50 μM Na vanadate, 7 mg ml^{-1} PNPP and 1 mM EDTA in 50 mM Tris–HCl, pH 7.0, for 30 min at room temp. (27°). The reaction was stopped by adding Laemmli's sample buffer [26]. The samples were then heated to 100° in a water bath for 5 min and subjected to denaturing PAGE, followed by autoradiography.

Autophosphorylation of protein kinases in SDS-polyacrylamide gels. Detection of autophosphorylating protein kinases in tissue extracts, after the resolution of proteins by SDS-PAGE and their subsequent denaturation and renaturation, was carried out according to ref. [19]. The sp. act. of [γ-^{32}P]ATP, used for the phosphorylation reaction, was 92.5 Bq pmol^{-1}.

Protein estimation. Protein was estimated by the method of ref. [27] using bovine serum albumin as a standard.

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