ACTA
PHYSIOLOGIAE
PLANTARUM
Vol. 20. No. 2. 1998: 119-122

Short communication

Changes in the activity of phosphoenolpyruvate carboxylating enzymes in germinating yellow lupin seeds

Wiktoria Ratajczak, Władysław Polcyn, Teresa Lehmann, Lech Ratajczak

Department of Plant Physiology, A. Mickiewicz University, Al. Niepodległości 14, 61-713 Poznań, Poland

Key words: *Lupinus luteus*, PEP carboxylase, PEP carboxykinase, seed germination

Abstract

The rate of phosphoenolpyruvate carboxylation by extracts from germinating lupin seeds was measured through the $H^{14}CO_3$ fixation. PEP carboxylation in seed axes increased during their imbibition, mainly as a result of the increase in the activity of PEP carboxylase [EC 4.1.1.31]. However, the activity of PEP carboxykinase [EC 4.1.1.38], present during the first 3 hours of imbibition, as well as the activity of PEP-carboxykinase [EC 4.1.1.49], after 24 hours of imbibition, have also been shown. Possible physiological role of the changes in the activity of PEP carboxylases during lupin seeds germination is discussed.

List of abbreviations: OAA, oxaloacetate; PEP, phosphoenolpyruvate

Introduction

The CO_2 fixation by PEP-carboxylating enzymes takes place in various plant tissues. Among the various functions of PEP carboxylase [EC 4.1.1.31] and PEP carboxykinase [EC 4.1.1.49] perhaps the most important is photosynthetic CO_2 fixation in C_4 and CAM plants (Andreo *et al.* 1987). Several further functions have been described for PEP carboxylase from C_3 plants (Latzko and Kelly 1983). The phosphoenolpyruvate carboxykinase [EC 4.1.1.49] catalyses a key step in the conversion of fats to sugars during the germination of fat-storing seeds, in which, its activity closely parallel the gluconeogenic flax (Leegood and Rees 1978, Bryce and Hill 1993, Walker *et al.* 1995). Another enzyme which exhibits activity in the early stages of seeds germination is PEP carboxykinase (pyrophosphate) [EC 4.1.1.38] (Perl 1978, 1986). PEP carboxylase in lupin was investigated mainly in root nodules (Marczewski 1989), where it plays a key role in the pathway of the synthesis of asparagine, which is transported from the nodule to other plant parts. During vegetative growth in lupin, CO_2 fixation and PEP carboxylase activity was correlated with biological nitrogen fixation (Christeller *et al.* 1977). Because in germinating lupin seeds, similarly to root nodules, there occurs an intense synthesis of asparagine (Lever and Butler 1971) it seems interesting to investigate whether PEP carboxylase plays a physiological role in asparagine synthesis also during seed germination. Our report presents the results of preliminary research into this area.

Material and methods

Seeds of *Lupinus luteus* L. *cv.* Ventus, S-elite class, were obtained from Plant Experiment Station in Wiatrowo. The seeds were imbibed on moistened

filter paper in Petri dishes, in darkness at the temperature of 24 °C. Embryonic seedlings axes were excised from the seeds after the 3rd, 12th and 24th hours of imbibition. Extraction and purification of soluble protein fractions were conducted as earlier described (Ratajczak *et al.* 1997). Protein content in the fractions were determined by the method of Bradford (1976) using BSA as the standard.

Enzyme assay

The activity of PEP carboxylating enzymes were assayed by the modified Holdsworth and Bruck (1977) method. The standard method used was to convert the oxaloacetate formed into acid stable malate by means of malate dehydrogenase in the presence of NADH („trapping system"). The reaction mixture contained in a final volume 1 ml: Tris/HCl buffer, (pH 8.0) 50 mM; dithiothreitol 1 mM; $MgCl_2$ 5 mM; $NaH^{14}CO_3$ 10 mM (5 mCi); $NADH_2$ 0.3 mM and 2 units of malate dehydrogenase. After temperature equlibration to 25 °C, the reaction was started by the addition of 2 mM phosphoenolpyruvate. The assay was stopped after 10 min with 0.5 ml of 1 N HCl and the sample was boiled for 5 min in the water bath. The acid stable radioactivity was quantitated in the presence of 5 ml Aquasol scintillation fluid (New England Nuclear) using a Packard counter. From the known concentration and radioactivity of the $NaH^{14}C0_3$ added, the number of nanomols of $H^{14}CO_3$ incorporated into acid-stable radioactivity in the vial was calculated.

Results and discussion

The fixation of CO_2 into oxalacetate could theoretically be performed by four known enzymatic mechanisms:

(1)
PEP carboxylase [EC 4.1.1.31],
reaction: $PEP + CO_2 + H_2O \rightarrow Pi + OAA$

(2)
PEP carboxykinase (GTP) [EC 4.1.1.32],
reaction: GDP (or ADP) + PEP + $CO_2 \rightarrow$ GTP (or ATP) + OAA

(3)
PEP carboxykinase (pyrophosphate) [EC 4.1.1.38],
reaction: $Pi + PEP + CO_2 \rightarrow PPi + OAA$

(4)
PEP carboxykinase (ATP) [EC 4.1.1.49],
reaction: $ADP + PEP + CO_2 \rightarrow ATP + OAA$

The reaction catalysed by PEP carboxylase does not require additional substrates, therefore the sign of this activity were the results obtained in variant 1 (Table). All the tested extracts showed PEP carboxylase activity increasing during lupin seeds germination. PEP carboxylase is a key enzyme of the pathway providing carbon skeletons in the form of oxaloacetate for amino acid synthesis, especially of asparagine (Tomaszewska *et al.* 1988). In our earlier paper (Ratajczak *et al.* 1990) we have demonstrated that an increase in asparagine level already takes place from the sixth hour of germination. It is

Table. Effect of various compounds on PEP-carboxylating activity. Extracts from germination lupin seed axes were noted as described by Ratajczak *et al.* 1997. The rate of carboxylation was assayed and calculated as described under „Material and methods". Values are means ± SE of three replicates.

Variant	Additions	Fixation of $^{14}CO_2$ (nmol · min^{-1} · mg $protein^{-1}$)		
		Time of germination (hours)		
		3	**12**	**24**
1	None	20 ± 4	138 ± 11	289 ± 17
2	ATP (2mM)	19 ± 5	132 ± 14	271 ± 20
3	ADP (2mM)	17 ± 5	145 ± 10	384 ± 22
4	GTP (2mM)	18 ± 3	135 ± 12	260 ± 15
5	GDP (2mM)	16 ± 5	133 ± 10	308 ± 24
6	Pi (0.5mM)	32 ± 4	122 ± 12	257 ± 14
7	Pi (1 mM)	48 ± 7	118 ± 9	250 ± 14
8	Pi (2mM))	75 ± 8	97 ± 8	212 ± 10
9	Pi (2mM)+PPi (2mM)	12 ± 3	115 ± 7	218 ± 21

quite probable that in germinating lupin seeds, analogically as in lupin root nodules, the PEP carboxylase provides OAA for asparagine synthesis.

In the extract isolated from seeds after 3 hours of imbibition, the activity of PEP carboxylating enzyme was demonstrated (Table). The activity of the enzyme was also stimulated by inorganic phosphate (variants 6-8, Table). Furthermore, it has also been shown that this enzyme is inhibited by pyrophosphate (variant 9, Table). Taking into account the above it seems that the observed activity belongs to PEP carboxykinase [EC 4.1.1.38]. In the imbibited lupin seeds this enzyme can play the same role as the one described by Perl (1978, 1986) for various species. PEP carboxykinase is known as a key enzyme in the pathway of ATP synthesis in the early stage of seeds germination (Perl 1986). PEP carboxykinase [EC 4.1.1.38] may also be active in the later stage of lupin germination, but we were unable to shown it conclusively since it was masked by a high activity of PEP carboxylase [EC 4.1.1.31].

It is certain, that the activity of another PEP carboxykinase (ATP)[EC 4.1.1.49] was present in the later stages of germination. Moreover, this result can be support by observed increase in the carboxylation of PEP by means of ADP (variant 3). Thus, the question arises whether it is possible that this enzyme is involved in the utilization of lipid breakdown products. It is well known in yellow lupin seeds that lipid is not the major storage material (it constitutes around 5 % of seed fresh mass, Wojnowska *et al.* 1996). But in lupin seeds, lipids are mobilized relatively early (after 24 hours of germination) (Czosnowski *et al.* 1978). This fact is in agreement with the rapid activation of PEP carboxykinase (ATP) that we observed.

Our preliminary data suggest that PEP-carboxylating enzymes play various functions during lupin seeds germination. Further investigation carried out on better purified enzymes should bring about the results which will precisely define the physiological role of these enzymes during lupin seeds germination.

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Received July 23, 1997; accepted February 12, 1998