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Short communication

Improved procedures for extraction of lysine 2-oxoglutarate reductase/saccharopine dehydrogenase (LOR/SDH) enzyme from *Phaseolus vulgaris* cultivars

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Abstract Lysine is one of the most limiting essential amino acid in food crops consumed by humans. Excess lysine is catabolised via saccharopine by two consecutive enzymes, lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH), linked on a single bifunctional polypeptide. The activities of LOR and SDH were shown to be similar in three distinct *Phaseolus vulgaris* cultivars. The addition of bovine serum albumin (BSA) to buffers resulted in different responses among the crude, 0–7.5%, and 7.5–15% polyethylene glycol (PEG) 8000 extracts. Different responses were also observed when the activities of LOR and SDH were tested after extraction in the presence of phosphatase inhibitors. The addition of lysine to the growth medium increased the activity of LOR, but not SDH. The results observed add new information on the regulation of LOR/SDH in a legume plant species.

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INTRODUCTION

The essential amino acid lysine is synthesised in higher plants via a pathway starting with aspartate, that also leads to the formation of threonine, methionine, and isoleucine (Azevedo et al. 1997). Two enzymes linked on a single bifunctional polypeptide catalyse the first two steps of lysine degradation. Lysine 2-oxoglutarate reductase (LOR) first combines lysine and 2-oxoglutarate into saccharopine, whereas sacharopine dehydrogenase (SDH) converts saccharopine into α-aminoadipic semialdehyde and glutamate (Arruda et al. 2000). LOR/SDH has been isolated from plant tissues which allowed the characterisation of some physical and biochemical properties, kinetics and modulation of LOR activity by calcium, ionic strength, and phosphorylation (Gonçalves-Butruille et al. 1996; Kemper et al. 1998; Gaziola et al. 2000; Galili et al. 2001a; Lugli et al. 2002). Although LOR/SDH has been characterised in a few cereal crops (Azevedo & Lea 2001; Azevedo 2002), the purification and characterisation of LOR/SDH from a dicotyledonous plant species has only been carried out in detail in soybean and *Arabidopsis thaliana* (Galili et al. 2001a). Furthermore, several reports with distinct plant species, including *Phaseolus vulgaris* have provided direct evidence for the functional significance of lysine catabolism in regulating lysine accumulation in seeds (Brochetto-Braga et al. 1992; Brennecke et al. 1996; Gaziola et al. 1999; Galili et al. 2001a; Cunha Lima et al. 2003). Recent genetic, molecular, and biochemical evidence suggest that lysine synthesis and catabolism are regulated by novel concerted mechanisms (Galili et al. 2001a,b; Galili 2002).

Although the amount of information about lysine catabolism in cereal plant species has increased considerably in the last few years, in legume plant species very little is known. In this study, LOR/SDH was isolated from *P. vulgaris* and characterised for enzyme stability, the effects of phosphatase inhibitors and lysine.

MATERIALS AND METHODS

The seeds of *P. vulgaris* were surface sterilised by soaking in a 25% (v/v) sodium hypochlorite with continuous stirring for 10 min, and rinsing in 70% ethanol for 3 min. The seeds were then washed with plenty of sterile deionised water, germinated and grown in autoclaved vermiculite, in a dark room at 20°C, unless stated otherwise. Ten days after germination, or just after the cotyledonal leaves had emerged, plants were frozen in liquid nitrogen in the dark, separating leaves, roots, hypocotyls, and cotyledons, and kept frozen at –70°C for further analysis.

LOR/SDH was isolated from the different tissues of bean plants and from maize endosperm, used as control for LOR/SDH activities, as described previously by Cunha Lima et al. (2003). Three cultivars of *P. vulgaris* denominated 'Carioca 80', 'Black bean', and 'White bean' were used. The maize plants were grown in the field, harvested at 18 days after pollination (DAP) and stored at –70°C. All procedures were carried out at 4°C unless stated otherwise.

The plant tissue (15–30 g) was ground with pestle and mortar in liquid nitrogen. The powder was homogenised in 5:3 v/w of buffer A (50 m*M* sodium phosphate, pH 7.4, containing 1 m*M* DL-dithiothreitol and 15 m*M* benzamidine). The homogenate was centrifuged at 20 000*g* for 10 min to remove cell debris and the supernatant was brought to pH 5.5 with NaH_2PO_4. Polyethylene glycol (PEG) 8000 was added to a 7.5% concentration, the homogenate centrifuged at 20 000*g* for 10 min and the pellet resuspended in buffer B (50 m*M* Tris-HCl, pH 8.5, containing 1 m*M* DL-dithiothreitol). The supernatant was subjected to a further PEG precipitation to a final concentration of 15% and centrifuged at 20 000*g* for 10 min. The sedimented proteins were dissolved in buffer B (50m*M* Tris-HCl, pH 8.5, containing 1 m*M* DL-dithiothreitol).

The activity of LOR was measured spectrophotometrically in the direction of NADPH to $NADP^+$, at 30°C, by following the change in absorbance at 340 nm over 15 min, with appropriate adjustments for a lysine-free blank, as described by Gaziola et al. (1997). The activity of SDH was also measured spectrophotometrically by following the rate of substrate dependent reduction of NAD^+ to NADH, monitored at 30°C over a 15 min period, with appropriate adjustments for a saccharopine-free blank as described by Gaziola et al. (1997). Assay reactions were started by adding the plant extract and monitoring the rates of oxidation and reduction at 340 nm in a Beckman DU-65 spectrophotometer. Data obtained were transformed in total enzyme activity (unit per g of tissue) and in specific activity (unit per mg of protein). One unit of enzyme activity is defined as the amount of enzyme necessary to catalyse the oxidation of 1 nmol NADPH (LOR) or reduction of 1 nmol NAD^+ (SDH), for 1 min, at 30°C. Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein kit and bovine serum albumin (BSA) as the protein standard.

Hypocotyl tissue was extracted in two variations of buffer A, one exactly as described above and the other containing 2% (w/v) BSA to test the effect of BSA on enzyme stability. One aliquot was taken from crude extract for measuring enzyme activity. After adding 7.5% PEG 8000, the precipitated proteins were resuspended in buffer B with and without 2% (w/v) BSA. The supernatants were subject to a further precipitation with PEG 8000 (7.5–15% (w/v)) for 30 min and then centrifuged at 20 000*g* for 10 min at 4°C. The supernatants were discarded and the pellets resuspended in buffer B with and without 2% (w/v) BSA, and then used for enzyme activity determinations.

An experiment was designed to determine the decay of enzyme stability from different cultivars of *P. vulgaris*, comparing to maize LOR/SDH, which has been shown to be a very stable protein. The hypocotyl of etiolated bean plants from the different cultivars ('Carioca 80', 'White bean', and 'Black bean') and the 18 DAP maize endosperms were used. Fifteen g of tissue was homogenised in buffer A containing 2% (w/v) BSA and 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 20 000*g* for 10 min, the supernatant was filtered in several layers of gauze, the pH was adjusted to 5.5 with NaH_2PO_4 and the proteins were precipitated with 15% PEG 8000. The pellet was resuspended in buffer B and the activities of LOR and SDH were estimated at regular intervals during a 50-h period (the extract was maintained at 4°C

between assays). The same procedure was carried out with the maize enzyme extract. A curve of enzyme activity per time was plotted and the $t_{1/2}$ (time for loosing half of the maximum enzyme activity) was determined for each plant by fitting the curve to a linear regression using the Origin 5.0 statistical program.

The effect of phosphatase inhibitors was also determined. Using the hypocotyls tissue, LOR/SDH was extracted as described above adding sodium vanadate (Na_3VO_4) to the extraction buffer. The activities of LOR/SDH from crude, 7.5%, and 7.5–15% PEG 8000 extracts prepared in buffer A containing 0, 0.05, 0.15, and 0.25 m*M* Na_3VO_4 were determined. Similarly, the same experiment was carried out adding 0.1 and 1 m*M* EDTA to buffer A.

LOR/SDH response to lysine addition *in vitro* was also investigated. Four hundred seeds of *P. vulgaris* ('Carioca 80') surface sterilised as described above were germinated in autoclaved filter paper moistened in sterile deionised water, in the dark, at room temperature. After the emission of the first roots, the embryonary axes were removed, divided in two sets of 150 plants, and then transferred to culture medium containing micronutrients and macronutrients (50% (w/v) Murashige & Skoog (1962) Basal Salt Mixture-SIGMA, pH 5.4, added of 50% (w/v) Phytagel Plant/Gellan Gum agar substitute—SIGMA Chemical Co.) with and without the addition of 10 m*M* lysine. The plants were incubated for a further 72-h growth under constant irradiance (50 μmol m^{-2} s^{-1}) at 20°C. The plants were collected taking all tissues together (an average of 13 g per treatment), LOR/SDH enzyme protein was isolated as described above, and the activities measured in the crude extract and the two protein fraction extracts (precipitated with 7.5% and 7.5–15% PEG 8000).

RESULTS

The distribution of LOR/SDH activities was shown to be similar among all studied cultivars of *P. vulgaris* (Table 1). The total enzyme activity was higher in the leaves (15 units/g fresh mass), however, the specific activity on protein basis was higher in the hypocotyls (8 units per/mg protein), for all studied plants. The other tissues exhibited lower total LOR/SDH activities, whereas in roots LOR activity could only be detected at trace levels in the 'Carioca 80'. The activities of LOR/SDH in different tissues refer to the enzyme of etiolated *P. vulgaris* plants, collected just after the first cotyledonary leaf had emerged (10 days after germination).

BSA, which can act against lipids and fatty acids, impairing the proteins from complexion, was added to the LOR/SDH extraction and purification buffers with the purpose of protecting enzyme activity against the action of proteases, and to maintain higher enzyme stability. The two PEG precipitated fractions responded differently to the presence of albumin. In the protein fraction precipitated with 7.5% PEG and in the crude extract fraction, the BSA did not exert any effect on the total activities of LOR/SDH, but in the 7.5–15% PEG fraction, BSA induced a significant increase of up to 4-fold in the stability of both enzyme activities (Table 2).

Increasing concentration of EDTA in the extraction buffer resulted in different responses among the three LOR/SDH extracts (Table 3). In crude and 7.5–15% PEG extracts a reduction in the activity of LOR was observed, however, the opposite occurred in the 7.5% PEG fraction. A reduction in SDH activity was also verified in the 7.5–15% PEG fraction, whereas the crude and 7.5% PEG extracts exhibited an increase in SDH activity in 0.1 m*M* EDTA, followed by a decrease in SDH activity

Table 1 Distribution of lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH) total activities (units per g of fresh mass) in distinct tissues of three cultivars of *Phaseolus vulgaris* ('White bean', 'Black bean', and 'Carioca 80'. (ND, not detectable.)

P. vulgaris variety		Root	Leaf	Cotyledon	Hypocotyl
White bean	LOR	ND	13.69 ± 0.69	8.60 ± 4.08	2.67 ± 0.53
	SDH	ND	9.32 ± 0.27	4.30 ± 1.50	2.15 ± 0.06
Black bean	LOR	ND	18.97 ± 0.61	5.38 ± 0.23	8.05 ± 0.61
	SDH	ND	8.67 ± 0.34	10.74 ± 4.50	6.13 ± 0.03
Carioca 80	LOR	1.05 ± 0.29	15.48 ± 0.99	3.87 ± 0.77	9.29 ± 0.13
	SDH	ND	14.51 ± 2.31	1.81 ± 0.28	6.03 ± 0.50

Table 2 Effect of bovine serum albumin (BSA) addition to the extraction buffer on lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH) total activities (units per g of fresh mass) in three hypocotyl extracts from *Phaseolus vulgaris* 'Carioca 80': crude extract, 7.5% polyethylene glycol (PEG) fraction, and 7.5–15% PEG fraction.

Extract		–BSA	+2% BSA
Crude	LOR	132.25 ± 8.51	112.90 ± 12.30
	SDH	81.55 ± 1.83	88.44 ± 4.82
7.5% PEG	LOR	53.76 ± 4.30	49.48 ± 2.80
	SDH	8.60 ± 0.50	16.49 ± 2.00
7.5–15% PEG	LOR	3.54 ± 0.30	18.81 ± 1.20
	SDH	4.37 ± 0.32	25.67 ± 2.10

Table 3 Effect of EDTA addition to the extract buffer on the total activities (units per g of fresh mass) of lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH) in crude extract, 7.5% polyethylene glycol (PEG) fraction and 7.5–15% PEG fraction, extracted from *Phaseolus vulgaris* 'Carioca 80'.

		EDTA concentration		
Extract		0	0.1 m*M*	1 m*M*
Crude	LOR	161.29 ± 16.91	120.97 ± 25.20	129.57 ± 15.31
	SDH	86.02 ± 6.17	94.35 ± 10.26	74.09 ± 14.10
7.5% PEG	LDH	40.33 ± 9.17	46.33 ± 8.50	46.82 ± 4.14
	SDH	8.05 ± 0.09	9.98 ± 0.46	6.93 ± 0.13
7.5–15% PEG	LOR	3.99 ± 0.18	2.15 ± 0.03	2.01 ± 0.11
	SDH	4.98 ± 0.73	3.34 ± 0.21	2.79 ± 0.33

Table 4 Effect of the phosphatase inhibitor vanadate (Na_3VO_4) on the total activities (units per g of fresh mass) of lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH) in crude extract, 7.5% polyethylene glycol (PEG) fraction and 7.5–15% PEG fraction, extracted from *Phaseolus vulgaris* 'Carioca 80'.

		Na_3VO_4 (m*M*)			
		0	0.05	0.15	0.25
Crude	LOR	107.53 ± 5.90	96.77 ± 11.90	49.46 ± 2.93	53.76 ± 3.77
	SDH	69.29 ± 7.62	82.26 ± 13.68	86.56 ± 3.50	88.71 ± 11.40
7.5% PEG	LOR	79.84 ± 2.50	69.99 ± 4.71	36.99 ± 1.21	36.13 ± 2.13
	SDH	9.58 ± 0.19	21.67 ± 0.94	6.36 ± 0.48	8.88 ± 1.20
7.5–15% PEG	LOR	14.03 ± 2.20	32.02 ± 1.79	0	0
	SDH	42.87 ± 1.10	50.13 ± 0.87	49.51 ± 1.09	40.95 ± 3.36

below the control levels when 1 m*M* EDTA was added to the buffer (Table 3).

Antagonic responses were also observed when Na_3VO_4 was added to the buffer. The activity of LOR was strongly reduced in all protein extracts with the increase in Na_3VO_4 concentration, leading to a complete inhibition of activity in the 7.5–15% PEG extracts at a concentration of 0.15 m*M* (Table 4). On the other hand, SDH activity was shown to increase in the crude extracts and also in the 7.5–15% PEG extracts in the presence of up to 0.15 m*M* Na_3VO_4, dropping to the control level in 0.25 m*M* Na_3VO_4, whereas in the 7.5% PEG extracts SDH activity did not exhibit a clear trend (Table 4).

The activity of LOR/SDH in the hypocotyls of beans measured in distinct intervals during a 50-h period demonstrated that the $t_{1/2}$ (time the enzyme takes to lose half of its maximum activity) varied between 21 ('White bean') and 34 h ('Carioca 80') for LOR and 77 ('White bean') to 194 h ('Carioca 80') for SDH (Fig. 1), indicating that in *P. vulgaris* the enzyme stability of the reductase function is 3.6–5.8 times lower than the dehydrogenase function. Among the three varieties tested, the 'Carioca 80' bean exhibited the highest stability for both enzyme functions. The enzyme from maize endosperm exhibited 10–12-fold higher activity than the *P. vulgaris* enzyme, also exhibiting a $t_{1/2}$ of 175.4 h for LOR, which indicates higher enzyme stability (up to 5-fold higher), when compared to 'Carioca 80' beans. Furthermore, it was also observed that the relation between LOR and SDH $t_{1/2}$ was different in maize, since LOR activity was maintained for a longer period when compared to SDH activity ($t_{1/2}$ of 127.93 h), which is the opposite of what was observed in *P. vulgaris*.

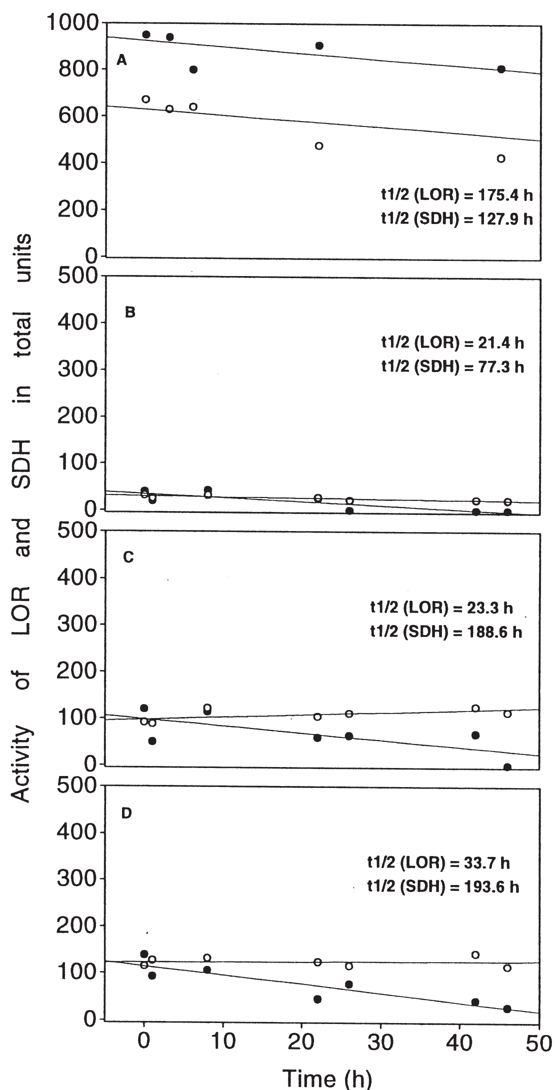

Fig. 1 Decay in enzyme activity according to time. Hypocotyl tissue from three cultivars of *Phaseolus vulgaris* and from immature endosperm of maize were used for enzyme extraction. They were homogenised in 50 m*M* sodium phosphate, pH 7.4. Proteins were precipitated with 15% polyethylene glycol 8000 and the pellets were resuspended in 50 m*M* Tris-HCl, pH 8.5. Curves were fitted to linear regression and the $t_{1/2}$ was determined to lysine 2-oxoglutarate reductase (LOR) (●) and saccharopine dehydrogenase (SDH) (○) activities.

Table 5 Lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH) activities (units/mg protein) in *Phaseolus vulgaris* 'Carioca 80' plants grown *in vitro*. All tissues were homogenised together and activities were measured in crude extract, and two protein fractions precipitated with 7.5% and 7.5–15% polyethylene glycol (PEG). Plants were grown in Murashige & Skoog medium with and without the addition of 10 m*M* lysine.

Extract		Control (–lysine)	+10m*M* lysine
Crude	LOR	1.79 ± 0.09	2.84 ± 0.11
	SDH	0.17 ± 0.03	0.24 ± 0.02
7.5% PEG	LOR	0.97 ± 0.45	2.18 ± 0.41
	SDH	0.77 ± 0.09	0.58 ± 0.03
7.5–15% PEG	LOR	6.91 ± 0.31	8.41 ± 0.80
	SDH	0.25 ± 0.01	0.57 ± 0.04

The presence of 10 m*M* lysine in the growing medium resulted in small increases of LOR activity in the 'Carioca 80' variety, in the crude extract and in both PEG precipitated protein fractions, when compared to the control grown without lysine (Table 5). On the other hand, lysine did not produce any major effect on the activity of SDH in all three extracts tested.

DISCUSSION

The metabolism of lysine has received a great deal of attention in the last few years, particularly with the isolation and characterisation of two enzymes, LOR and SDH, which present key roles in the regulation of lysine degradation. Recent research has focused mainly on the purification and characterisation of these enzymes in cereal seeds. Recently, these two enzymes were isolated and partially characterised in *P. vulgaris* (Cunha Lima et al. 2003).

It could be observed that LOR/SDH activity is higher in the hypocotyl tissue independent of the variety analysed. This is possibly a result of the amino acid pool that this plant species accumulates in its tissues. Isoleucine, leucine, and lysine are the essential amino acids normally observed in higher concentrations in the seeds of *P. vulgaris* (Blanco & Bressani 1991). Furthermore, there is a high demand for lysine from the seed in *P. vulgaris*, since this amino acid is found incorporated in storage proteins, especially in the globulin and albumin proteins. It is possible that a higher incorporation of lysine in the seed proteins is responsible for the low concentration of this amino acid in the soluble form, which could explain the reduced LOR/SDH activity in the seeds as previously reported (Cunha Lima et al. 2003). On the other hand, in maize seeds where the demand for lysine from the endosperm is very small (Gaziola et al. 1999), the soluble lysine concentration is maintained low as a result of the high LOR/SDH activities. Such a hypothesis is further supported by similar results observed in tissues from other plant species, such as tobacco leaves containing high lysine content associated to trace activity levels of LOR/SDH (Galili et al. 2001a).

LOR/SDH activities were also detected in *P. vulgaris* ('Carioca 80') plants grown *in vitro* from embryos, which had the cotyledons previously removed. Under this condition, the addition of lysine to the growing medium induced an increase in enzyme activity, particularly LOR, whereas SDH activity was decreased in the 7.5% PEG fraction, suggesting the existence of two distinct SDH isoenzymes. Such increases observed were quite low when compared to the increase observed for LOR activity of *Nicotiana tabacum* plants (Galili et al. 2001a). In that instance, induction of enzyme activity was obtained through the injection of lysine directly into the seeds. The different procedures adopted for tobacco and in this study, could partially explain the weak response verified in the *in vitro* bean plants. Another aspect is that with *P. vulgaris*, the whole plant was homogenised together and the enzyme was extracted from a mixture of all tissues, since the size of the *in vitro* bean plants was very small for organ separation. This could have probably diluted the effect on enzyme activity if compared to the effect that lysine could cause if hypocotyls had been analysed separately from the other tissues.

The 7.5% and 7.5–15% PEG-precipitated enzymes presented different responses to the addition of BSA to the extract. In a previous study (Cunha Lima et al. 2003), differences were also observed for protease inhibitors (aprotinin, PMSF, leupeptin, sodium fluoride) and a phosphatase inhibitor (sodium molybdate), suggesting the possibility of having two enzyme isoforms present in the hypocotyls, which could be separated by PEG fractioning. The isoform precipitated with 7.5% PEG is less affected by the addition of BSA, indicating a higher stability. Other plant enzymes have also been shown to respond to the addition of proteins to the extraction medium. For instance, the presence of casein (Santoro & Magalhães 1983), increased significantly the half-life of nitrate reductase because of a stabilising effect on the enzyme activity. This result indicates that LOR/SDH belonging to 7.5–15% PEG fraction is more susceptible to BSA protection, maintaining a higher enzyme conformation when BSA is added to the buffer. Moreover, it is also possible that proteases, lipids, and fatty acids may be acting strongly on LOR/SDH precipitated in this protein fraction. In crude extracts, such as for the 7.5% PEG fraction, LOR/SDH total activities were not affected by BSA probably because this fraction contains the majority of LOR/SDH activities.

In this study, EDTA was used to diminish the protein dephosphorylation. Addition of EDTA was shown to inhibit LOR and SDH activities, particularly in the 7.5–15% PEG fraction, suggesting

that a change in the phosphorylated state of the protein occurred or that both enzyme activities are dependent upon ions that were chelated by EDTA. Increasing concentrations of Na_3VO_4 in the extraction buffer produced different results between LOR and SDH activities and the PEG extracts, so that LOR activity was reduced significantly with the increase of Na_3VO_4 in all hypocotyl extracts, whereas a slight increase or no changes were observed for SDH activity. In a previous study (Cunha Lima et al. 2003) the addition of sodium molibdate resulted in improved LOR activity with a much smaller effect on SDH activity. Such antagonic results suggest that *P. vulgaris* LOR/SDH may be regulated by phosphorylation/dephosphorylation of serine-threonine residues. The results also confirm that LOR activity is the domain more strongly modulated, mainly by opposing actions of protein kinases and phosphatases (Cunha Lima et al. 2003). The combination of these results strongly support the suggestion for the presence of at least two distinct LOR/SDH proteins, possibly with the presence of a monofunctional SDH as has been previously demonstrated in arabidopsis and canola (Galili et al. 2001b).

The half-life of *P. vulgaris* enzyme was measured and compared to the LOR/SDH maize enzymes, which had been previously shown to be a stable polypeptide (Gonçalves-Butruille et al. 1996). The results revealed that, depending on the variety, the dehydrogenase function is 3–6-fold more stable than the reductase function in *P. vulgaris*, whose result is the opposite of what was observed for the maize enzymes, where LOR activity exhibits a longer half-life, reflecting a slightly better stability than SDH. Comparing the stability of maize LOR/SDH activities to those of the bean plants tested, although LOR activity was several-fold more stable in maize, the SDH activity of bean plants were on average more stable than the maize enzyme. This result, together with others obtained previously (Cunha Lima et al. 2003), indicate that addition of BSA and protease inhibitors to buffers, may be essential when purifying LOR/SDH activities from a dicotyledonous species, particularly because of the low activities and the losses observed during the purification of the enzymes (Cunha Lima et al. 2003).

The mechanisms controlling and regulating LOR and SDH activities appear to differ among tissues and plant species. Furthermore, the LOR/SDH polypeptide from dicotyledonous plants is also more sensitive to manipulation, suggesting the use of compounds to increase enzyme stability. Finally, the results also suggest the existence of different isoenzymes of LOR/SDH, with LOR domain being a phosphoprotein with the activity modulated by the opposing actions of protein kinases and phosphatases.

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