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Isolation of the bifunctional enzyme lysine 2-oxoglutarate reductase-saccharopine dehydrogenase from *Phaseolus vulgaris*

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Summary. Lysine is catabolyzed by the bifunctional enzyme lysine 2-oxoglutarate reductase-saccharopine dehydrogenase (LOR-SDH) in both animals and plants. LOR condenses lysine and 2-oxoglutarate into saccharopine, using NADPH as cofactor and SDH converts saccharopine into α-aminoadipate δ-semialdehyde and glutamic acid, using NAD as cofactor. The distribution pattern of LOR and SDH among different tissues of *Phaseolus vulgaris* was determined. The hypocotyl contained the highest specific activity, whereas in seeds the activities of LOR and SDH were below the limit of detection. Precipitation of hypocotyl proteins with increasing concentrations of PEG 8000 revealed one broad peak of SDH activity, indicating that two isoforms may be present, a bifunctional LOR-SDH and possibly a monofunctional SDH. During the purification of the hypocotyl enzyme, the LOR activity proved to be very unstable, following ion-exchange chromatography. Depending on the purification procedure, the protein eluted as a monomer of 91–94 kDa containing only SDH activity, or as a dimer of 190 kDa with both, LOR and SDH activities, eluting together.

Keywords: *Phaseolus vulgaris* – Catabolism – Lysine – Lysine 2-oxoglutarate reductase – Saccharopine dehydrogenase

1. Introduction

Human food and animal feed derived from many cereals are deficient in lysine (Azevedo and Lea, 2001). Due to this deficiency, there has been considerable interest in studying the metabolic pathways in which this amino acid is synthesized (Azevedo et al., 1997; Azevedo, 2002). One approach to raising the lysine content of seeds is to increase the rate of synthesis using transgenic plants (e.g. tobacco and canola), expressing lysine-insensitive aspartate kinase (AK) and/or dihydrodipicolinate synthase (DHPDS) (Falco et al., 1995; Brinch-Pedersen et al., 1996). These plants, however, displayed severe phenotypic alterations when the genes were expressed in the leaves (Azevedo and Lea, 2001; Galili et al., 2001).

Lysine catabolism is also an important mechanism for the control of the concentration of soluble lysine in maize endosperm (Azevedo et al., 1997; Azevedo and Lea, 2001). The first two steps of lysine degradation in plants and mammals are catalyzed by the bifunctional enzyme protein lysine 2-oxoglutarate reductase-saccharopine dehydrogenase (LOR-SDH) (also known as lysine α-ketoglutarate reductase-saccharopine dehydrogenase LKR-SDH) (Azevedo et al., 1997; Papes et al., 1999; Arruda et al., 2000; Molina et al., 2001). LOR and SDH from plants and animals have some similar properties, with a optimum pH of 7.0 for LOR activity and 8.5 for SDH (Fjellstedt and Robinson, 1975; Brochetto-Braga et al., 1992; Gaziola et al., 2000). In yeast and other fungi, these two activities correspond to the last step of the biosynthetic route for the formation of lysine and reside in two separate proteins (Markovitz and Chuang, 1987; Azevedo et al., 1997).

LOR and SDH apparently control important processes in all organisms, although their cellular function and modulation of activities vary among species. The presence of high concentrations of lysine in plant tissues frequently seems to be the result of the reduced catabolism of this amino acid. In transgenic tobacco leaves containing a deregulated DHDPS enzyme, with a high concentration of lysine, no detectable activity of LOR-SDH was found (Karchi et al., 1995). In the

opaque-2 mutant of maize, where the concentration of soluble lysine and lysine incorporated into the endosperm storage proteins are higher when compared to normal maize, enzymatic activities are reduced 2- to 10-fold (Brochetto-Braga et al., 1992; Kemper et al., 1999). Moreover, the quality protein maize (QPM) varieties, produced by the introduction of opaque-2 modifier genes, exhibited even lower levels of LOR and SDH activities when compared to the opaque-2 maize mutant (Gaziola et al., 1999).

LOR-SDH was partially purified from rice (Gaziola et al., 1997; 2000) and the data indicated the existence of a dimeric bifunctional polypeptide of 202 kDa and another of 396 kDa, possible constituents of a multimeric structure. The enzymes have also been studied in *Coix lacryma-jobi* (Lugli et al., 2002) and in the dicotyledonus plants *Arabidopsis thaliana* (Tang et al., 1997) and *Glycine max* (Miron et al., 1997; 2000). In *A. thaliana*, a bifunctional enzyme, with LOR and SDH activities and a monofunctional enzyme, with only SDH activity, were identified (Tang et al., 1997), whereas in *G. max* and *C. lacryma-jobi* these two enzyme activities co-purified during all steps of purification (Miron et al., 2000; Lugli et al., 2002).

The molecular and biochemical regulation of lysine catabolism is still not clearly understood. In maize, where this mechanism has been studied in most detail, the native form of the enzyme is a dimer of 260 kDa (Gonçalves-Butruille et al., 1996) and the LOR domain is activated by Ca^{2+}, high saline concentration, organic solvents and Mg^{2+} (Kemper et al., 1998; 1999). Very similar results have been reported for the rice LOR-SDH bifunctional polypeptide (Gaziola et al., 2000). Studies with *G. max* also demonstrated that phosphorylation is essential for LOR activity but is not involved in the regulation of SDH activity, suggesting that these linked activities are differentially regulated by post-translational modifications (Miron et al., 1997). Evidence for the phosphorylation control of LOR activity has also been obtained in tobacco (Karchi et al., 1995). Other evidence suggesting differential regulation comes from the demonstration that the SDH domain is able to inhibit LOR activity *in vitro* (Kemper et al., 1998). In rapeseed leaf discs, lysine catabolism was also shown to be osmoregulated at the level of LOR and SDH (Moulin et al., 2000). Recent results have shown that *A. thaliana* LOR possess catabolic activity, but not anabolic activity, with a unidirectional activity related to its structure rather than its linkage to the SDH domain (Zhu et al., 2000).

In the present work, the lysine catabolism of *Phaseolus vulgaris* has been studied by the characterization of the bifunctional enzyme LOR-SDH in different tissues of this plant species.

2. Material and methods

2.1. Plant material

Seeds of *Phaseolus vulgaris* cv. Carioca 80 were supplied by the Instituto Agronômico de Campinas (IAC) and grown in the dark at 25 ± 2°C. All tissues from *P. vulgaris* were collected in the dark, 10 days after germination. Maize endosperms (17 days after pollination) were used as control for LOR and SDH activities. All plant materials were harvested and stored at −70°C prior to enzyme extraction.

2.2. Enzyme extraction

LOR-SDH was extracted as previously described by Gonçalves-Butruille et al. (1996), from root, seed, pod, leaf, cotyledon and hypocotyl of *P. vulgaris*, and from the maize endosperm. The subsequent steps were carried out at 4°C. 15 g of each tissue were homogenized in 25 ml of buffer A (sodium phosphate 50 mM, pH 7.4, containing 1 mM DL-dithiothreitol, 1 mM EDTA and 15 mM benzamidine). The homogenate was centrifuged at 20,000 g for 10 min and the supernatant was brought to pH 5.5 by the addition of NaH_2PO_4. Polyethylene glycol (PEG) 8000 was added to a final concentration of 15%, the samples were centrifuged at 20,000 g for 10 min and the pellet resuspended in buffer B (50 mM Tris-HCl, pH 8.5, 1 mM DL-dithiothreitol and 1 mM EDTA). Using the hypocotyl tissue, different extraction conditions were subsequently adopted, including fractional precipitation with PEG 8000, addition of protease or phosphatase inhibitors to the buffers, as indicated in the figure legends.

2.3. Enzyme assays

LOR activity was measured spectrophotometrically in the direction of NADPH oxidation at 30°C by following the change in absorbance over a 15 min period, with appropriate adjustments for a lysine-free blank. The reaction mixture had a final volume of 0.8 ml and contained 20 mM L-lysine, 10 mM 2-oxoglutarate (neutralized to pH 7.0 with potassium hydroxide), 0.1 mM NADPH and 175 mM Tris HCl, pH 7.4. 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, in a reaction mixture with a final volume of 0.8 ml containing 1 mM saccharopine, 0.4 mM NAD and 100 mM Tris-HCl (pH 8.5). The assay reactions were started by adding the plant extract and the rates of oxidation and reduction were monitored at 340 nm in a Beckman DU-65 spectrophotometer. Assays were performed with and without the compound to be tested in the blank, to ensure that any possible interference was being taken into account. The protein concentration of all enzyme extracts was determined by the method of Bradford (1976) using the Bio-Rad Protein Assay dye reagent. One unit of enzyme activity was defined as 1 nmol of NADPH oxidized or NAD reduced min^{-1} at 30°C.

2.4. Partial purification of LOR-SDH

LOR-SDH was extracted (1 : 2 w/v) from the hypocotyls of etiolated plants as described previously for other tissues (see enzyme extrac-

tion), according to established methods (Markovitz and Chuang, 1987; Gonçalves-Butruille et al., 1996) with few modifications. The protease inhibitors phenylmethylsulphonyl fluoride (PMSF) (0.5 mM), aprotinin (50 μM), leupeptin (100 μM) and BSA (2%, w/v) were added to the buffer A. The protein fractions precipitated with 7.5% and 7.5–15% PEG 8000 were separately used for enzyme purification following the same procedure. After precipitation with PEG, the samples were centrifuged at 20,000 g for 10 min, dialyzed overnight in buffer B and applied to a DEAE-Sephacel ion exchange chromatography column. Enzymes were eluted with a 0–500 mM NaCl linear gradient and the fractions containing LOR-SDH activity were combined and precipitated with 70% $(NH_4)_2SO_4$. After centrifugation at 20,000 g for 10 min, the pellets were resuspended in buffer B, dialyzed against an excess of the same buffer, applied to a Protein Pak-Q 8HR ion exchange chromatography column and eluted with 0–500 mM NaCl linear gradient. Fractions containing LOR-SDH activities were pooled and applied to a Superdex 200 HR gel filtration column previously equilibrated with buffer B, containing 300 mM NaCl. Enzyme activity was monitored in all steps of purification. For gel filtration determinations of the approximate molecular mass of the enzyme, the following molecular mass markers were used: thyroglobulin (669 kDa), apoferritin (440 kDa), catalase (232 kDa), BSA (67 kDa) and ovalbumin (43 kDa). The full purification procedure was repeated 20 times over a 3 year period and the data presented is a representative sample of one such purification.

2.5. SDS-PAGE

Electrophoresis was carried out in order to determine the apparent molecular mass under denaturing conditions, exactly as described by Laemmli (1970). 4.0 μg of enzyme, from the different steps of purification, were applied to a 8% polyacrylamide SDS gel.

3. Results

3.1. Distribution pattern of enzyme activity among the tissues of P. vulgaris

Crude extracts of root, seed, pod, leaf, cotyledon and hypocotyl of *P. vulgaris* were prepared as described in the Experimental section. Activities of crude extract and polyethylene glycol (PEG) fractions were monitored and compared to extracts of maize endosperm prepared in the same way. The highest LOR and SDH specific activities were found in the hypocotyl (Fig. 1), with LOR activity being higher than SDH activity. The specific activity of LOR in roots, pods and leaves proved to be low (approximately 2.0 units · mg^{-1} protein), but even lower in the cotyledons (1 unit · mg^{-1} protein) and below the limit of detection in seeds (Fig. 1). The specific activity of SDH was again below the detectable limits in roots and seeds, and was very low in pods but increased in cotyledons and leaves and reached the highest level in the hypocotyl as observed for LOR (Fig. 1). The specific activities of maize endosperm LOR and SDH after isolation with PEG 8000 (97.8 and 21.8 units · mg^{-1} protein, respectively),

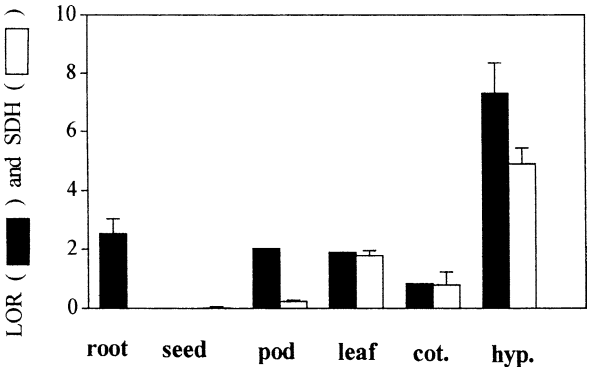

Fig. 1. LOR and SDH activites in different tissues of *P. vulgaris*. Tissues were homogenized, clarified, and proteins were concentrated with 15% PEG 8000. The bars represent the standard error (n = 3). Enzyme activity expressed in units · mg^{-1} protein

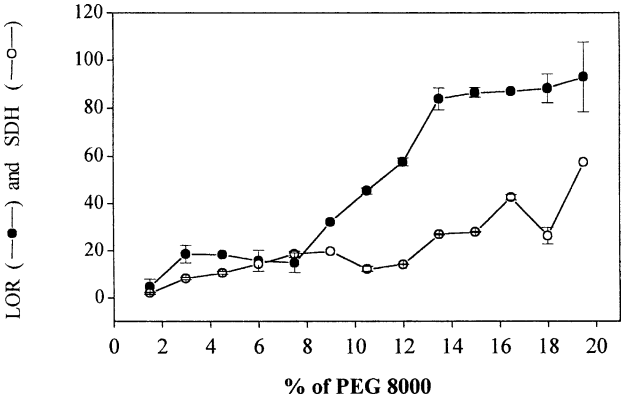

Fig. 2. Activity of LOR and SDH following the precipitation of proteins from hypocotyl extracts of *P. vulgaris* with increased concentrations (expressed as a %) of PEG 8000. The bars represent the standard error (n = 3). Enzyme activities are expressed in units · mg^{-1} protein

were around 11- and 4-fold higher, respectively, than that exhibited by *P. vulgaris* hypocotyls.

The distribution of the two enzyme activities in the various tissues revealed a similar pattern in the white-bean and black-bean varieties (data not shown), both exhibiting the highest specific activity in hypocotyl extracts. Based on these results, this tissue was chosen for the isolation and characterization of LOR-SDH.

3.2. PEG fractions revealed one broad peak of SDH activity

The preparations obtained after fractional precipitation with PEG were used to determine LOR and SDH activities. LOR activity was higher in all PEG precipitated extracts except between 6.0–7.5% PEG, when the activities were approximately the same (Fig. 2). At

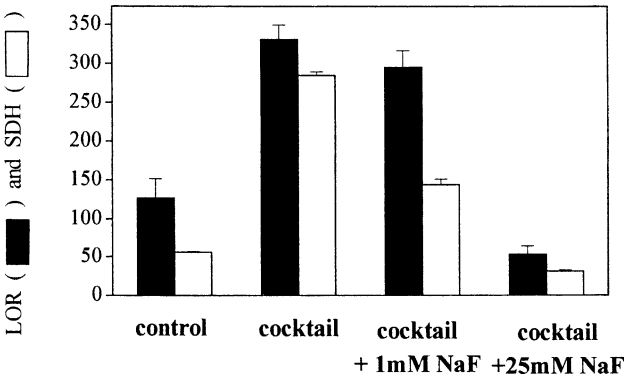

Fig. 3. The effect of protease inhibitors on the isolation of LOR-SDH activity from *P. vulgaris* hypocotyls. Extraction was carried out in buffer A (*control*); in buffer A containing a cocktail of protease inhibitors (50 μM aprotinin, 100 μM leupeptin and 0.5 mM PMSF); and in buffer A containing the inhibitor cocktail plus the addition of 1 or 25 mM NaF. Proteins were concentrated with 15% PEG after the pH was brought to 5.5 with NaH_2PO_4. Samples were centrifuged and the pellet resuspended in buffer B. The bars represent the standard error (n = 2). Enzyme activities expressed in units · mg^{-1} protein

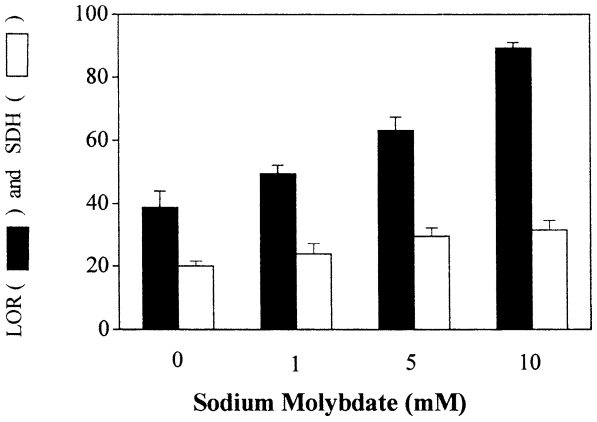

Fig. 4. The effect of the protease inhibitor, sodium molybdate on the activity of LOR and SDH isolated from *P. vulgaris* hypocotyls. Buffer A contained 2% (w/v) BSA and increasing concentrations of sodium molybdate and after precipitation with PEG, the pellet was taken up in buffer B containing the same concentrations of sodium molybdate. Activity was determined for the 0–7.5% PEG fraction. The bars represent the standard error (n = 3). Enzyme activities expressed in units · mg^{-1} protein

concentrations of PEG higher than 7.5%, there was a dramatic increase in the activity of LOR and to a lesser extend of SDH.

3.3. Presence of protease inhibitors in hypocotyl extracts prevented LOR and SDH activity losses

Extraction of LOR and SDH in buffer A, with the addition of the protease inhibitors aprotinin (100 μM), PMSF (0.5 mM) and leupeptin (50 μM) resulted in higher LOR and SDH activities, particularly for SDH (Fig. 3). However, the addition of sodium fluoride (NaF) at concentrations of 1 and 25 mM inhibited both LOR and SDH activities, with the 25 mM concentration leading to LOR and SDH activities lower than the control without the cocktail of protease inhibitors (Fig. 3).

3.4. LOR is activated by a phosphatase inhibitor

Increasing concentrations of sodium molybdate in buffers A and B resulted in improved LOR activity with a much smaller effect on SDH (Fig. 4). This result was obtained with the 7.5% PEG fraction, which was shown to be more stable in the previous experiment, when BSA was added to the buffer system.

3.5. Purification of LOR-SDH from the hypocotyl tissue

The LOR-SDH activities were partially purified from *P. vulgaris* hypocotyls. Very high losses of LOR activity were observed during purification (Table 1). On the other hand, although SDH specific activity was drastically reduced after the gel filtration step, a significant increase in SDH specific activity was observed up to the Protein Pak Q purification step (Table 1).

In the final stages, LOR activity could not be measured due to a total loss of activity, usually during the dialysis or ion exchange chromatography step. The addition of $CaCl_2$ or glycerol to the enzyme mixture did not restore or stabilize LOR activity.

3.6. Apparent molecular mass

The native molecular mass of the enzyme was determined on a Superdex 200HR gel filtration column, whose elution profile is shown for both PEG fractions (0–7.5% and 7.5–15%) in Figs. 5 and 6. The peaks of SDH activity were both estimated to have a molecular mass of 94 kDa.

The preparation obtained for each step of enzyme purification was subject to SDS-PAGE. A band of 91 kDa was gradually enriched and became predominant in the gel filtration fraction (Fig. 7). However,

Table 1. Purification steps of LOR/SDH from hypocotyl tissue of *P. vulgaris*

Fraction	LOR				SDH			
	Total protein	Total activity	Specific activity	Yield	Total activity	Specific activity	Yield	LOR/SDH
	mg	*nmol. min^{-1} · ml^{-1}*	*unit/mg*	%	*nmol. min^{-1} · ml^{-1}*	*unit/mg*	%	
Crude extract	425.6	7237.0	17.0	100	5065.9	11.9	100	1.42
PEG 7.5–15%	222.7	1138.06	5.1	15.7	1667.9	7.48	32.9	0.682
Dialysed DEAE	10.21	50.9	4.98	0.7	221.5	21.7	4.37	0.22
Protein Pak Q	0.88	0	0	0	134.5	152.8	2.65	∞
Superdex 200HR	3.36	0	0	0	56.7	16.8	1.11	∞

One unit of enzyme activity is defined as the amount of enzyme necessary to oxidise 1 nmol of NADPH or reduce 1 nmol of NAD, per min, at 30°C

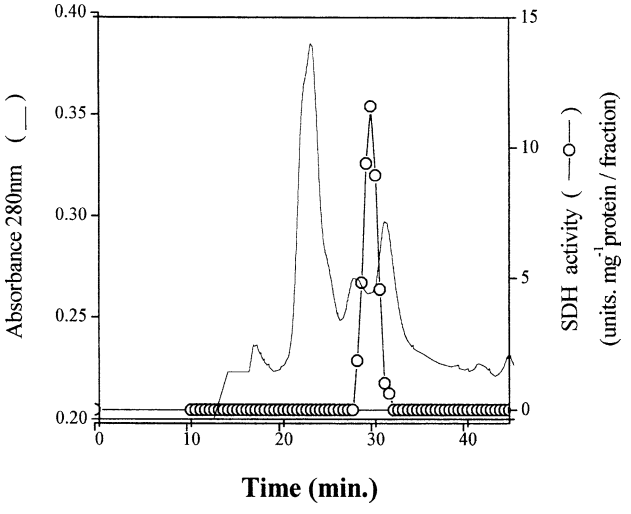

Fig. 5. Elution profile of SDH from the Superdex 200HR gel filtration column (0.5 ml/min flow rate; 0.3 ml fraction size). A 0–7.5% PEG fraction was used. Activity expressed in units · mg^{-1} protein

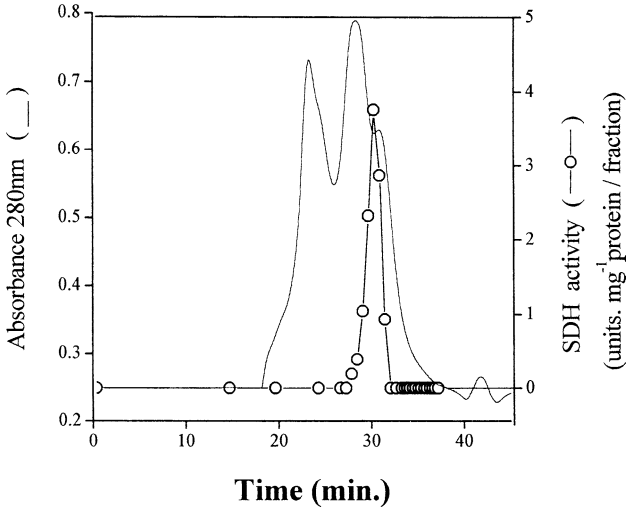

Fig. 6. Elution profile of SDH from the Superdex 200HR gel filtration column (0.5 ml/min flow rate; 0.3 ml fraction size). A 7.5–15% PEG fraction was used. Activity expressed in units · mg^{-1} protein

other contaminant bands of lower molecular mass were still present after the final step of enzyme purification. A LOR-SDH maize endosperm partially purified sample applied to the same gel confirmed a molecular mass of around 125 kDa for the maize enzyme and was also used as a reference control for the *P. vulgaris* enzyme molecular mass determination (Fig. 7, lane 5). Further confirmation of the 91 kDa molecular mass of *P. vulgaris* SDH was obtained when the enzyme activity containing fractions eluted from the Superdex 200HR column were pooled and concentrated 9-fold in Amicon 30 and subject to a SDS-PAGE (Fig. 8).

Although the ratio LOR/SDH varied during purification and LOR activity was not detectable in the last two stages, LOR and SDH activities co-eluted in all other chromatographic steps which may suggest the presence of a bifunctional LOR/SDH polypeptide in *P. vulgaris*, as previously observed for other plants species (Gonçalves-Butruille et al., 1996; Gaziola et al., 1997; Tang et al., 1997). Since LOR activity was always lost in the ion exchange chromatography step, in some experiments the PEG step was substituted by ammonium sulphate precipitation (35–70% saturation) and after a desalting step on Sephadex G25, the sample was loaded directly onto the Superdex 200 HR column. This procedure resulted in a different elution profile of SDH activity (Fig. 9). The peak of SDH activity also contained LOR activity and the molecular mass was estimated as 190 kDa.

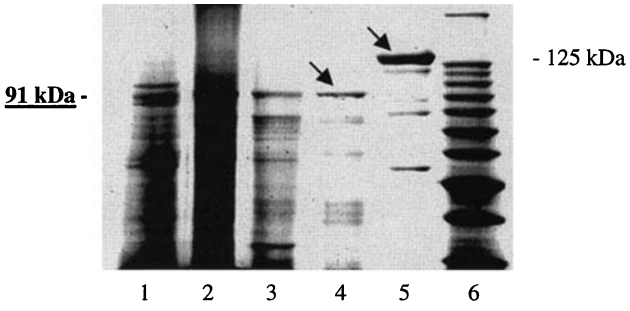

Fig. 7. SDS-PAGE of enzyme extracts from each step of purification and comparison with LOR-SDH from maize endosperm. Lane *1*, crude extract; lane *2*, 7.5% PEG fraction; lane *3*, enzyme eluted from the DEAE-Sephacel column; lane *4*, enzyme eluted from the Superdex 200 HR gel filtration column; lane *5*, maize LOR-SDH and lane *6*, 10 kDa protein ladder (GIBCO-BRL) molecular mass marker. 4 μg of enzyme were applied to each lane in a 8% polyacrylamide gel. Coomassie Blue was used for staining. The bands of 91 kDa and 125 kDa indicated with an arrow, are possibly the *P. vulgaris* and *Zea mays* LOR/SDH enzyme protein, respectively

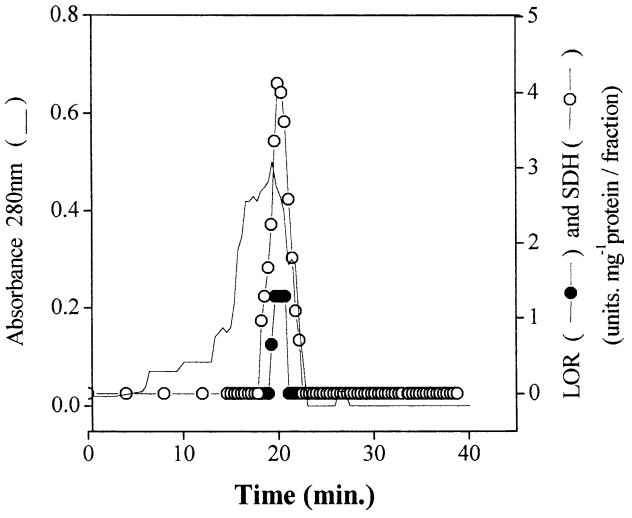

Fig. 9. Elution of LOR and SDH activity isolated from *P. vulgaris* hypocotyls, from Superdex 200HR gel filtration step, using a 35–70% ammonium sulphate fraction

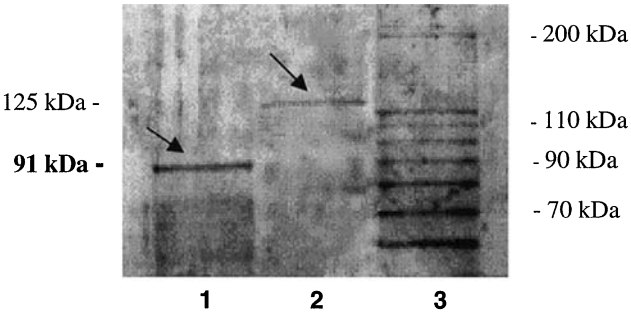

Fig. 8. SDS-PAGE of LOR-SDH partially purified from *P. vulgaris*, concentrated in Amicon 30. Lane *1*, the arrow indicates the enzyme purified from the hypocotyls of *P. vulgaris* (concentrated 9-fold); lane *2*, the same enzyme from maize (without previous concentration); lane *3*, 10 kDa protein ladder (GIBCO-BRL) molecular mass marker. 4 μg were applied to each lane and Coomassie Blue was used for staining

4. Discussion

Lysine catabolism has received considerable attention recently, due to the novel findings on the characterization and regulatory aspects of the bifunctional enzyme LOR-SDH (Azevedo and Lea, 2001). In this study, the bifunctional enzyme LOR-SDH was isolated from different tissues of *P. vulgaris* and partially purified and characterized from the hypocotyl. The enzyme activities were higher in hypocotyl extracts of etiolated plants (Fig. 1), although these activities were very low when compared to the other plants species studied so far (Gonçalves-Butruille et al., 1995; Gaziola et al., 1997; Miron et al., 1997; Gaziola et al., 1999). The requirement of lysine for the synthesis of globulins and albumins (Blanco and Bressani, 1991) may underlie the lack of LOR-SDH activity in the seeds of *P. vulgaris*. The mechanism is the opposite of that which occurs in maize (Brochetto-Braga et al., 1992), where the main seed storage protein is zein, a prolamin with a low concentration of lysine, which would require a higher rate of lysine breakdown. In the maize mutant opaque-2, which exhibits a reduction in the prolamin storage protein and simultaneous increase in the other storage protein fractions, the rate of lysine degradation is reduced, allowing a higher amount of lysine to be incorporated into the storage proteins or to be present in the soluble form (Azevedo et al., 1990; Brochetto-Braga et al., 1992; Gaziola et al., 1999; Arruda et al., 2000). Moreover, although rice is also limited in lysine like all other cereal crops (Teixeira et al., 1998; Lugli et al., 2000), among these groups of plants species, the rice seed contains the higher concentration of lysine due to a distinct distribution of the storage protein fractions, indicating a higher requirement of lysine such as the opaque-2 mutant of maize (Azevedo et al., 1990). In a similar way, this mutant exhibits a much lower rate of lysine breakdown (Gaziola et al., 1997; Gaziola et al., 2000; Azevedo and Lea, 2001). It is likely that legume crops, due to the higher concentration of lysine, exhibit a reduced rate of LOR-SDH activity to allow lysine to be accumulated into storage proteins or in the soluble form. Further confirmation for such regulatory pattern of lysine

catabolism has been obtained in soybean and canola which accumulated lysine in the seeds, but although LOR or SDH activities have not been tested, intermediates of lysine catabolism also accumulated in the seeds of these plants, suggesting the presence of reduced LOR and/or SDH activities (Falco et al., 1995).

Both LOR and SDH activities were detected in a range of tissues of *P. vulgaris*, which differs from previous data obtained with cereal crops, in which the activities of LOR-SDH have only been detected in the seeds (Azevedo and Lea, 2001). LOR activity proved to be equal or higher than SDH, depending on the tissue analyzed (Fig. 1). In the hypocotyl, LOR had a greater specific activity than SDH, as previously shown for maize (Gonçalves-Butruille et al., 1996), rice (Gaziola et al., 1997) and soybean (Miron et al., 1997) seeds. Nevertheless, in the liver of most animals studied (Markovitz and Chuang, 1987; Papes et al., 1999) the SDH activity is higher, reflecting an enzyme whose activity varies among species and tissues.

The concentration of protein from hypocotyl extracts with PEG 8000, revealed the presence of one broad peak of SDH activity and clear evidence of increased LOR activity at the higher PEG concentrations (Fig. 2), which could suggest the existence of at least two LOR-SDH isoforms. Recent reports in rice (Gaziola et al., 2000), maize (Kemper et al., 1999) and *A. thaliana* (Tang et al., 1997) have suggested the occurrence of a monofunctional SDH enzyme, which may also be the case in *P. vulgaris*. In rice, several bands of SDH activity have been observed following native PAGE, suggesting a multimeric structure for the LOR-SDH enzyme, but also indicating the possibility of a monofunctional SDH polypeptide (Gaziola et al., 2000). LOR activity was shown to be very unstable, in particular during the ion exchange chromatography step, which is usually employed for the purification of LOR-SDH from other plants. This result suggested that this step is possibly causing disruption of the dimeric state of the protein, which appears to be a necessary condition for LOR activity. As discussed previously, in plants LOR-SDH is mainly a dimer, with each polypeptide containing LOR and SDH domains (Arruda et al., 2000; Azevedo and Lea, 2001; Azevedo, 2002). This fact raises the hypothesis that during the purification procedure, the polypeptide lost the dimerized state and eluted from the gel filtration column as a monomer with half the size of the native enzyme (91–94 kDa) and containing only SDH activity. The results with the ammonium sulphate fraction also showed that both, LOR and SDH activities, co-eluted during the purification procedure and that the bifunctional polypeptide has an approximate molecular mass of 190 kDa.

The use of phosphatase inhibitors has significantly contributed to the understanding of the biological processes which are controlled by reversible protein phosphorylation (Smith and Walker, 1996). In tobacco (Karchi et al., 1995), treatment of wild-type seed with okadaic-acid triggered a significant induction in LOR activity, while in soybean (Miron et al., 1997), *in vitro* dephosphorylation of the bifunctional polypeptide with alkaline phosphatase significantly inhibited LOR activity, but not the activity of the linked enzyme SDH. Some other reports also revealed that serine-threonine protein phosphatases found in the thylakoid membranes of different plant species were inhibited by sodium molybdate (Bennet, 1980; Cheng et al., 1995). Such an inhibition of serine-threonine protein phosphatases by sodium molybdate was responsible for maintaining the enzyme in its phosphorylated state, possibly necessary for the activity of the LOR domain in the hypocotyl enzyme. This phosphatase inhibitor was used in the present work and was shown to increase the LOR activity of the more stable LOR-SDH isoform of the hypocotyl. This could indicate that LOR in *P. vulgaris* is a phosphoprotein and that the activity is modulated by the opposing actions of protein kinases and phosphatases. Furthermore, the maize (Kemper et al., 1998), rice (Gaziola et al., 2000) and tobacco (Karchi et al., 1995) enzymes were shown to be modulated by calcium, and in some cases also by ionic strength (Kemper et al., 1998; Gaziola et al., 2000).

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References

Arruda P, Kemper EL, Papes F, Leite A (2000) Regulation of lysine catabolism in higher plants. Trends Plant Sci 5: 324–330

Azevedo RA (2002) Analysis of the aspartic acid metabolic pathway using mutant genes. Amino Acids 22: 217–230

Azevedo RA, Lea PJ (2001) Lysine metabolism in higher plants. Amino Acids 20: 261–279

Azevedo RA, Arana JL, Arruda P (1990) Biochemical genetics of the interaction of the lysine plus threonine resistant mutant Ltr*19 with opaque-2 maize mutant. Plant Sci 70: 81–90

Azevedo RA, Arruda P, Turner WL, Lea PJ (1997) The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. Phytochemistry 46: 395–419

Bennet J (1980) Chloroplast phosphoproteins: evidence for a thylakoid-bound phosphoprotein phosphatase. Eur J Biochem 104: 85–89

Blanco A, Bressani R (1991) Amino acids bioavailability in beans (*Phaseolus vulgaris*). Archivos Latinoamericanos de Nutricion 41: 38–52

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72: 248–254

Brinch-Pedersen H, Galili G, Knudsen S, Holm PB (1996) Engineering of the aspartate family biosynthetic pathway in barley (*Hodeum vulgare* L.) by transformation with heterologous genes encoding feedback-insensitive aspartate kinase and dihydrodipicolinate synthase. Plant Mol Biol 32: 661–620

Brochetto-Braga MR, Leite A, Arruda P (1992) Partial purification and characterization of lysine ketoglutarate reductase in normal and opaque-2 maize endosperms. Plant Physiol 98: 1139–1147

Cheng L, Stys D, Allen JF (1995) Effects of synthetic peptides on thylakoids phosphoproteins. Physiol Plant 93: 173–178

Falco SC, Guida T, Locke M, Mauvais J, Sanders C, Ward RT, Weber P (1995) Transgenic canola and soybean seeds with increased lysine. Biotechnology 13: 577–582

Fjellstedt TA, Robinson JC (1975) Purification and properties of L-lysine-α-ketoglutarate reductase from human placenta. Arch Biochem Biophys 168: 536–548

Galili G, Tang GL, Zhu XH, Karchi H, Miron D, Gakiere B, Stepansky A (2001) Molecular genetic dissection and potential manipulation of lysine metabolism in seeds. J Plant Physiol 158: 515–520

Gaziola SA, Teixeira CMG, Lugli J, Sodek L, Azevedo RA (1997) The enzymology of lysine catabolism in rice seeds. Isolation, characterization, and regulatory properties of a lysine 2-oxoglutarate reductase/saccharopine dehydrogenase bifunctional polypetide. Eur J Biochem 247: 364–371

Gaziola SA, Alessi ES, Guimarães PEO, Damerval C, Azevedo RA (1999) Quality protein maize: biochemical study of enzymes involved in lysine metabolism. J Agric Food Chem 47: 1268–1275

Gaziola SA, Sodek L, Arruda P, Lea PJ, Azevedo RA (2000) Degradation of lysine in rice seeds: effect of calcium, ionic strength, S-adenosylmethionine and S-2-aminoethyl-L-cysteine on the lysine 2-oxoglutarate reductase-saccharopine dehydrogenase bifunctional enzyme. Physiol Plant 110: 164–171

Gonçalves-Butruille M, Szajner P, Torigoi E, Leite A, Arruda P (1996) Purification and characterization of the bifunctional enzyme lysine ketoglutarate reductase – saccharopine dehydrogenase from maize. Plant Physiol 110: 765–771

Karchi H, Miron D, Ben-Yaacov S, Galili G (1995) The lysine dependent stimulation of lysine catabolism in tobacco seed requires calcium and protein phosphorylation. Plant Cell 7: 1963–1970

Kemper EL, Cord-Neto G, Capella AN, Gonçalves-Butruille M, Azevedo RA, Arruda PA (1998) Structure and regulation of the bifunctional enzyme lysine ketoglutarate reductase – saccharopine dehydrogenase in maize. Eur J Biochem 253: 720–729

Kemper EL, Cord-Neto G, Papes F, Martinez Moraes KC, Leite A, Arruda P (1999) The role of opaque 2 in the control of lysine-degrading activities in developing maize endosperm. Plant Cell 11: 1981–1993

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lugli J, Campbell A, Gaziola SA, Smith RJ, Lea PJ, Azevedo RA (2002) Enzymes of lysine metabolism from *Coix lacryma-jobi* seeds. Plant Physiol Biochem 40: 25–32

Lugli J, Gaziola SA, Azevedo RA (2000) Effects of calcium, *S*-adenosylmethionine, *S*-(2-aminoethyl)L-cystein, methionine, valine and salt concentration on rice asparate kinase isoenzymes. Plant Sci 150: 51–58

Markovitz PM, Chuang DT (1987) The bifunctional aminoadipic semialdeyde synthase in lysine degradation. J Biol Chem 262: 9353–9358

Miron D, Ben-Yaacov S, Karchi H, Galili G (1997) In vitro dephosphorylation inhibits the activity of soybean lysine-ketoglutarate reductase in a lysine regulated manner. Plant J 12: 1453–1458

Miron D, Ben-Yaacov S, Reches D, Schupper A, Galili G (2000) Purification and characterization of bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase from developing soybean seeds. Plant Physiol 123: 655–663

Molina SMG, Gaziola SA, Lea PJ, Azevedo RA (2001) Manipulating cereal crops for high lysine. Scientia Agricola 58: 205–211

Moulin M, Deleu C, Larher F (2000) L-lysine catabolism is osmoregulated at the level of lysine-ketoglutarate reductase and saccharopine dehydrogenase in rapeseed leaf discs. Plant Physiol Biochem 38: 577–585

Papes F, Kemper EL, Cord-Neto G, Langone F, Arruda P (1999) Lysine degradation through the saccharopine pathway in mammals: involvement of both bifunctional and monofunctional lysine-degrading enzymes in mouse. Biochem J 344: 555–563

Smith RD, Walker JC (1996) Plant protein phosphatases. Ann Rev Plant Mol Biol 47: 101–125

Tang G, Miron D, Zhu-Shimoni JX, Galili G (1997) Regulation of lysine catabolism through lysine-ketoglutarate reductase and saccharopine dehydrogenase in *Arabidopsis*. Plant Cell 9: 1305–1316

Teixeira CMG, Gaziola SA, Lugli J, Azevedo RA (1998) Isolation, partial purification and characterization of aspartate kinase isoenzymes from rice seeds. J Plant Physiol 153: 281–289

Zhu XH, Tang GL, Galili G (2000) The catabolic function of the alpha-aminoadipic acid pathway in plants is associated with unidirectional activity of lysine oxoglutarate reductase, but not saccharopine dehydrogenase. Biochem J 351: 215–220

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