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Isolation and Characterization of Enzymes Involved in Lysine Catabolism from Sorghum Seeds

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Lysine is an essential amino acid synthesized in plants via the aspartic acid pathway. The catabolism of lysine is performed by the action of two consecutive enzymes, lysine 2-oxoglutarate reductase (LOR, EC 1.5.1.8) and saccharopine dehydrogenase (SDH, EC 1.5.1.9). The final soluble lysine concentration in cereal seeds is controlled by both synthesis and catabolism rates. The production and characterization of high-lysine plants species depends on knowledge of the regulatory aspects of lysine metabolism and manipulation of the key enzymes. We have for the first time isolated, partially purified, and characterized LOR and SDH from developing sorghum seeds, which exhibited low levels of activity. LOR and SDH were only located in the endosperm and were very unstable during the isolation and purification procedures. LOR and SDH exhibited some distinct properties when compared to the enzymes isolated from other plant species, including a low salt concentration required to elute the enzymes during anion-exchange chromatography and the presence of multimeric forms with distinct molecular masses.

KEYWORDS: Aspartate kinase; lysine; lysine 2-oxoglutarate reductase; maize; saccharopine dehydrogenase

INTRODUCTION

Cereal protein is a major component of the total human protein consumption throughout the world, and in addition, an increasing number of cereal crops are being used to feed animals. Sorghum grain is the staple food for people living in the semiarid regions of Africa and Asia and is also a major animal feed grain, used both in developed and in developing countries (*1*). With the identification of the high lysine mutant gene (*hl*) (*2*), new perspectives for improving the nutritional quality of sorghum grain have opened up.

Plants are able to synthesize all 20 amino acids that are incorporated into proteins (*3*, *4*). However, monogastric animals can only synthesize 11, and the remaining essential amino acids must therefore be obtained from the diet. Since cereal seeds constitute the main source of plant protein and are characterized by a deficiency in lysine, tryptophan, and threonine, detailed studies of the aspartate metabolic pathway in which lysine and threonine are synthesized have been carried out. These studies have lead to a better understanding of the metabolism of lysine and threonine and have provided information for the genetic manipulation of plants to improve the nutritional value of cereal seeds (*5*, *6*).

The multicellular nature of higher plants introduces additional levels of complexity to the regulation of amino acid metabolism that render metabolic fluxes much more difficult to predict and engineer when compared to microorganisms, for which metabolic engineering of amino acids has been successfully achieved (*7*). The synthesis of the essential amino acids lysine, threonine, methionine, and isoleucine are carried out in a complex and strongly regulated metabolic pathway, which has aspartic acid as a precursor with several enzymes being regulated by feedback inhibition (*5*). Several research groups have focused attention on understanding the biochemical and genetic controls of the aspartate pathway, inducing and selecting for lysine and threonine overproducing plants through genetic manipulation of the key steps catalyzed by the enzymes aspartate kinase (AK, EC 2.7.2.4), homoserine dehydrogenase (HSDH, EC 1.1.1.3), dihydrodipicolinate synthase (DHDPS, EC 4.1.2.52), threonine synthase (TS; EC 4.2.3.1), lysine 2-oxoglutarate reductase (LOR, EC 1.5.1.8), and saccharopine dehydrogenase (SDH, EC 1.5.1.9) (*7*−*12*). Mutants containing an AK insensitive to lysine feedback

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inhibition and transgenic tobacco plants expressing a lysine-insensitive AK from *Escherichia coli* exhibited overproduction and accumulation of threonine but had no significant changes in the soluble lysine concentration in the seeds (*13*−*15*). Other transgenic plants constructed with altered enzyme regulation combining AK and DHDPS did not exhibit any significant lysine accumulation in the seeds (*16*, *17*). Azevedo et al. (*18*−*20*) showed that increased lysine content in some maize mutants could be attributed to a lower rate of lysine catabolism by reduced endosperm LOR and SDH activities, reduced prolamine, and increased nonzein protein content and alterations in the regulation of the enzymes involved in lysine biosynthesis.

Although extensive information is available about the biosynthetic enzymes such as AK, DHDPS, and HSDH, until recently very little was known about lysine degradation (*21*−*25*). Recent studies have indicated that lysine catabolism plays an important role in lysine accumulation in plants and the control of lysine content, particularly of seeds (*22*, *23*). The lysine catabolic pathway in plants is carried out by LOR, which condenses lysine and 2-oxoglutarate to form saccharopine, which is then hydrolyzed to α-aminoadipic acid and glutamic acid in a reaction catalyzed by the enzyme SDH (*23*, *26*). Monofunctional LOR and SDH and bifunctional LOR−SDH polypeptides have been observed in plants (*27*−*29*). In maize (*27*), rice (*30*), and coix (*31*), the bifunctional enzyme LOR−SDH was shown to be endosperm-specific. Lysine was also shown to be able to regulate its own catabolism in vivo, with the enzymes LOR and SDH being differentially modulated by an intracellular signaling cascade, involving mainly Ca^{2+}, protein phosphorylation−dephosphorylation, and ionic strength (*32*−*34*).

Regulatory mechanisms controlling lysine metabolism are still not fully understood, and some hypotheses have been suggested. Arruda et al. (*22*) and Galili et al. (*35*) reported alternative hypotheses, which considered the linkage between LOR and SDH domains responsible for LOR activity modulation through protein intramolecular interactions. Zhu et al. (*36*) showed that the functional interaction is mediated by the linker region and not by specific affinities between these domains.

Azevedo and Lea (*6*) suggested that lysine overproduction and accumulation in cereal seeds might be obtained by combining the genetic manipulation of the biosynthesis and degradation mechanisms. This could be explained by the fact that plants including the maize opaque-2 mutant, which contain higher concentrations of soluble lysine in the seeds, exhibit a reduction in the lysine catabolic rate in the endosperm, thus allowing excess lysine to be incorporated into storage proteins, as well as increasing the accumulation in the soluble form (*23*, *37*). Further information may be obtained by investigating in more detail the expression and synthesis of storage proteins in high-lysine mutants, such as the research carried out with the opaque and floury maize mutants (*18*−*20*). Success in obtaining high-lysine plants for human or animal consumption relies on the full understanding of the metabolism of lysine as well as for subsequent genetic manipulation (*21*, *23*). We report here the first detailed study of lysine catabolism in sorghum with the isolation and partial characterization of LOR and SDH from developing seeds.

MATERIALS AND METHODS

Plant Material. Immature seeds of the sorghum (*Sorghum bicolor* (L.) Moench) commercial line Massa 03 (Dow Agroscience) were used for extraction of the enzymes and amino acids. The seeds were harvested at three distinct stages of development: 93, 97, and 100−101 days−stage 1, 2 (milk), and 3, respectively. Plants were grown in the field agricultural area of the Escola Superior de Agricultura Luiz de Queiroz, in the summer season of 2001, which exhibited a normal temperature and precipitation values for the season, but irrigation was maintained at least once a week. The developing seeds were harvested directly into liquid nitrogen and stored at −80 °C for further analysis. Other sorghum lines (accessions IS 11758, IS 11167, IS 16210, IS 10477, IS 22204, IS 5603) used for Infratec analysis were kindly donated by the International Crop Research Institute for Semi Arid and Tropics (ICRISAT) India. These mature seeds were previously stored in a chamber maintained at 15 °C and 45% humidity for a period of at least 3 days before analysis.

Enzyme Extraction. All procedures were carried out at 4 °C. Immature seeds were used for enzyme extraction. LOR and SDH were extracted from frozen seeds in five volumes of buffer as described by Gaziola et al. (*30*) with some modifications [15% glycerol (v/v), 10% PVPP (w/v)]. The extracts were filtered through several layers of gauze and centrifuged at 15 000*g* for 30 min to completely remove the cellular debris.

Ammonium Sulfate Precipitation. The centrifuged initial extract was submitted to sequential precipitation exactly as described by Lugli et al. (*31*). The supernatant was divided in two fractions and was precipitated by solid ammonium sulfate slowly being added and gently stirred for 30 min to give precipitation sequences of 0−20, 20−40, 40−60, 60−80% and 0−30, 30−50, and 50−70% ammonium sulfate saturation. After each step, the suspension was centrifuged at 15 000*g* for 30 min, and the precipitated protein dissolved in a small volume of buffer A (100 mM Tris-HCl pH 7.4, containing 1 mM DTT, 1 mM EDTA, and 10% (v/v) glycerol). The dissolved pellets were then loaded onto Sephadex G25 columns (10 mL total volume) equilibrated in buffer A and run under gravity. The desalted samples were assayed for LOR and SDH activities.

LOR and SDH Partial Purification. The enzymes were extracted as described previously and partially purified with 30−70% ammonium sulfate saturation. After the resuspended protein pellets were desalted on Sephadex G25 columns as described previously, the extracts were used in all experiments and for further purification steps. Rice LOR and SDH enzymes were used as a control of enzyme activity and were partially purified with 25−45% ammonium sulfate saturation as described by Gaziola et al. (*30*).

An anion exchange chromatography step was performed for the enzymes. Both stepwise and linear gradient types of elution were tested. The desalted samples were applied to a DEAE-Sephacel column (2.5 × 15 cm; flow rate 1 mL min^{-1}) equilibrated in buffer A. The column was washed with buffer A (unbound fraction—stored at −20 °C for further analysis), and a stepwise elution (0−100, 100−200, 200−300, 300−400, and 400−500 mM KCl) was carried out. A final wash with 1 M KCl was also performed, and all the fractions were stored at −20 °C for further analysis of enzyme activity. Similarly, another sample was eluted using a gradient of 0−250 mM KCl from the same column and same running conditions. The linear gradient eluted fractions containing LOR−SDH activity were combined and applied to a gel filtration Superose 12HR 10/30 column, connected to an Akta Purifer System (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The column was equilibrated, protein elution was carried out in buffer A (flow rate of 0.5 mL min^{-1}), and fractions (0.5 mL) were immediately assayed for LOR activity.

Enzyme Assays. Enzyme activities were assayed as described previously by Azevedo et al. (*18*), with a minor alteration (100 μL of extract were added to the reaction mixture). LOR/SDH activities were expressed as nmol of $NADPH/NAD^+$ oxidized/reduced min^{-1} mg^{-1} of protein. SDH activity staining was performed using nondenaturing PAGE gels as described by Gaziola et al. (*29*). A maize SDH enzyme sample partially purified was also applied to the gels and used as control of SDH activity and for enzyme comparison. Twenty μg of protein was applied to each gel lane.

Protein Determination. The protein concentration of all samples was determined by the Bradford method (*38*) using bovine serum albumin as a standard.

Amino Acid Extraction and Analyses. Soluble amino acids were extracted from immature sorghum seeds, essentially as described by Bieleski and Turner (*39*). Immature seeds were weighed, extracted in

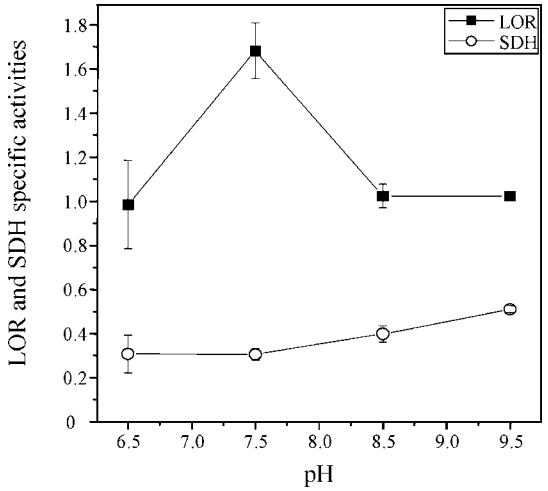

Figure 1. Determination of optimum pH for sorghum LOR and SDH activities. An ammonium sulfate partially purified LOR and SDH sample from immature seeds was used in the enzymatic assays. Enzyme activities are expressed in nmol of NADPH min^{-1} mg^{-1} of protein for LOR and in nmol of NAD min^{-1} mg^{-1} of protein for SDH ± SE.

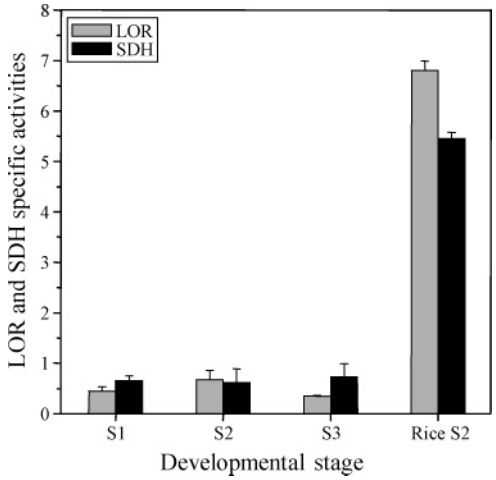

Figure 2. LOR and SDH activities in sorghum seeds at three developmental stages and in developing rice seeds (milk S2 stage). The activities are expressed in nmol of NADPH oxidized min^{-1} mg^{-1} of protein for LOR and nmol of NAD reduced min^{-1} mg^{-1} of protein for SDH.

five volumes of methanol/chloroform/water (12:5:3, v/v/v) and then centrifuged at 2500*g*. The pellet was submitted to hydrolysis in 6 M HCl for 22 h at 110 °C, before amino acid analysis. The samples were centrifuged, and the supernatant was stored at 4 °C. Soluble and total amino acids samples were quantified as described by Yemm and Cocking (*40*) and were separated by reverse-phase HPLC as described by Gaziola et al. (*37*), using the OPA derivatization method. A Spherisorb ODS-2 C18 column was eluted with 0.8 mL min^{-1} flux to form a linear gradient with solutions of 65% methanol and phosphate buffer, pH 7.5 (50 mM sodium acetate, 50 mM disodium phosphate, 1.5 mL of acetic acid, 20 mL of tetrahydrofuran, and 20 mL of methanol in 1 L of water). A standard solution containing all amino acids was derivatized with OPA and utilized for column profile and elution sequence identification. Each amino acid analysis was performed with three replicates using independent preparations.

Infrared Analyses. The infrared absorbance transmission analyses were performed in a Feed and Food 1255 Infratec Analyzer (Foss Tecator AB, Hönas, Sweden). Twenty-three mature seeds of each sorghum line were analyzed in each step of the programmed analysis, and the results were expressed as means. Three replicates were analyzed for the mutants and normal genotypes (mutants: IS 11758, IS 11167, IS 16210, IS 10477, IS 22204, IS 5603; wild-type: Massa 03).

RESULTS AND DISCUSSION

Optimum pH. We have isolated and studied the two key enzymes that control lysine catabolism in higher plants from sorghum seeds. Some of the properties reported for the enzymes isolated from other plant species have also been observed in sorghum, although some of them appear to be specific for the sorghum enzymes. For instance, the optimum pH (**Figure 1**) values for LOR (7.5) and SDH (higher than 8.5) were similar to those already reported for other species, such as maize (*27*) and rice (*30*), a characteristic that appears to vary very little in higher plants.

Tissue Specificity. Tissue specificity analysis indicated that LOR and SDH activities could only be detected in immature seeds (data not shown), confirming previous results obtained from others cereal seeds (e.g., maize (*27*), rice (*30*), and coix (*31*)), in which the activities of LOR and SDH have also only been detected in the developing seed. Such specificity appears to be a characteristic of cereal crops since legume crops have shown different results (e.g., in *Phaseolus vulgaris*, LOR and/or SDH activities were detected in roots, pods, leaves, cotyledons, and hypocotyls but below the limit of detection in seeds (*41*, *42*)). The result obtained in sorghum further confirms that lysine degradation via the saccharopine pathway is more important in cereal seeds when compared to legume seeds. Therefore, immature sorghum seeds were used for further partial purification and characterization.

Partial Purification. The sorghum LOR and SDH activities were not significantly different among all three seed developmental stages tested (**Figure 2**). In other cereals such as rice (*30*) and maize (*37*), the intermediate stages of development exhibited higher enzyme activity. LOR and SDH activities were mainly recovered within the range of ammonium sulfate saturation (**Figure 3A**) from which these enzymes have been detected in other plant species (*30*, *37*). Such a concentration range also precipitated the highest amount of total protein from sorghum seeds (**Figure 3B**). The ammonium sulfate saturation ranges required to isolate the enzymes have been shown to vary among the plant species studied so far, being 25−50% for rice (*30*) and 35−60% for maize (*43*), indicating that the majority of the enzyme activities can generally be isolated in the range of 40−50% ammonium sulfate saturation. LOR and SDH activities expressed on a protein basis recovered from sorghum seeds were also shown to be very low when compared to any of the other cereal crop species analyzed so far in the literature (*18*−*20*, *22*, *26*, *30*, *31*, *37*) and are also observed in **Figure 2**.

Several protein separation methods were tested to obtain a high purification of LOR and SDH enzymes from stage 2 immature sorghum seeds (data not shown); however, purification of the enzymes had to be limited to an ammonium sulfate precipitation and an anion exchange chromatography step (**Table 1**). This was due to the high enzyme instability after the ammonium sulfate precipitation step, which normally led to major or even complete loss of both enzyme activities even after several variations to the buffer systems were tested, including the addition of several different protease inhibitors. Although a high degree of purification of these enzymes has been obtained for the majority of the plant species studied so far, for a few, including cereal crops such as coix, enzyme activity was extremely unstable (*31*). The purification of LOR and SDH from *P. vulgaris* has also been difficult for similar reasons, although LOR activity was considerably more sensitive than SDH activity to in vitro manipulation (*41*, *42*). Some positive results were obtained with the addition of BSA, which can prevent proteins

Table 1. Purification of LOR and SDH Enzymes from Immature Sorghum Seeds[a]

step	total protein (mg)	total activity (NADPH/NAD oxidized/reduced min^{-1})		specific activity (NADPH/NAD oxidized/reduced min^{-1} mg^{-1} of protein)		purification (fold)		yield (%)	
		LOR	SDH	LOR	SDH	LOR	SDH	LOR	SDH
crude extract	2260								
ammonium sulfate	1838	1681	778	0.91	0.42				
DEAE-Sephacel	6.56	140	29.8	21.34	4.54	23.45	10.8	8.33	3.83

[a] LOR and SDH use NADPH and NAD as cofactor, respectively.

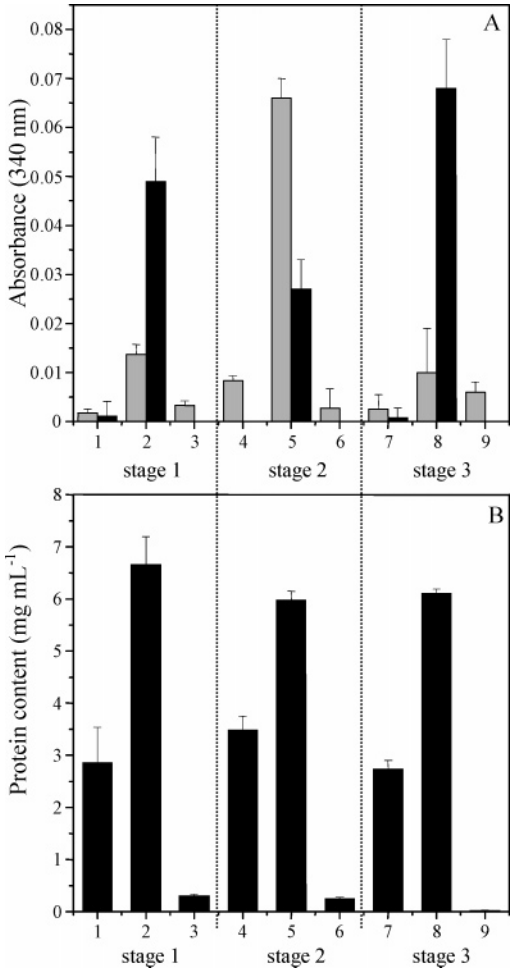

Figure 3. Determination of the ammonium sulfate precipitation range of LOR and SDH in three developmental stages of the seed (100 g of each as starting material). In **panel A**, LOR (gray box) and SDH (black box) activities (absorbance at 340 nm) and in **panel B**, protein content for each ammonium sulfate saturation step. Stages 1–3 correspond to the three seed developmental stages defined as early, intermediary, and later, respectively, and the ammonium sulfate saturation steps 1, 4, and 7 = 0–40%; 2, 5, and 8 = 40–70%; and 3, 6, and 9 = 70–80%.

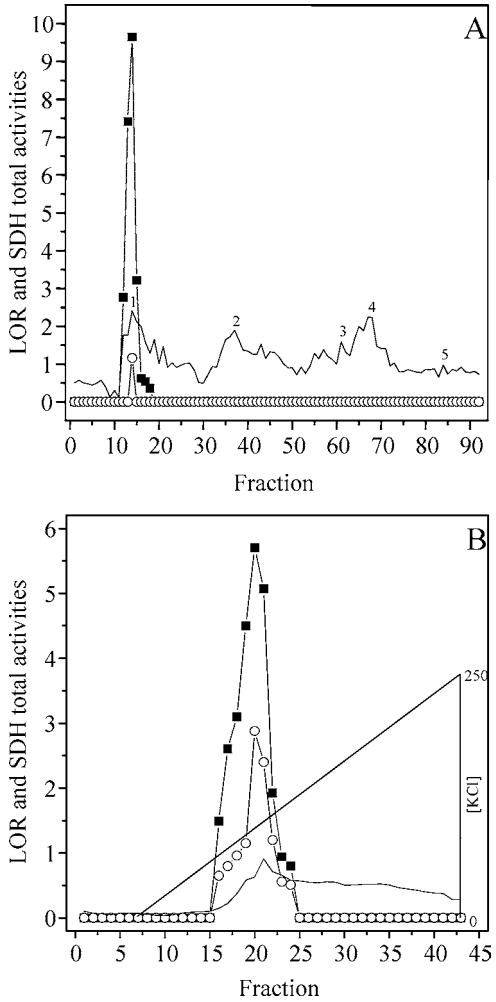

Figure 4. Elution profile of LOR and SDH enzymes from a DEAE-sephacel anion exchange chromatography column. In **panel A**, stepwise elution system (1–100 mM; 2–200 mM; 3–300 mM; 4–400 mM; 5–500 mM KCl). In **panel B**, linear gradient elution system (0–250 mM KCl). Protein elution profile was determined at 280 nm (·····), and LOR (■) and SDH (○) total activities are expressed in nmol of NADPH oxidized min^{-1} and nmol of NAD reduced min^{-1}, respectively.

from complexing with lipids and fatty acids and with higher EDTA concentrations, suggesting that a change in the phosphorylated state of the enzymes occurred or that both enzymes activities are dependent upon ions that were chelated by EDTA (*42*). Phosphatase inhibitors such as vanadate and sodium molybdate also exhibited distinct effects on LOR and SDH activity, further confirming the occurrence of different states of phosphorylation of these enzymes but with LOR being more strongly modulated than SDH activity in plants (*41*, *42*).

Initially, a stepwise elution from the DEAE-Sephacel column was attempted with both enzymes being totally eluted at 100 mM KCl, while the other concentrations of KCl did not elute any LOR or SDH activity (**Figure 4A**). When the samples were eluted from the same column with a 0−250 mM KCl linear gradient (**Figure 4B**), only one peak of LOR and SDH activity was eluted at approximately 80 mM KCl, confirming the data obtained in the stepwise elution. The KCl concentration used for the elution of sorghum LOR−SDH was much lower than the concentrations previously reported for the elution of these

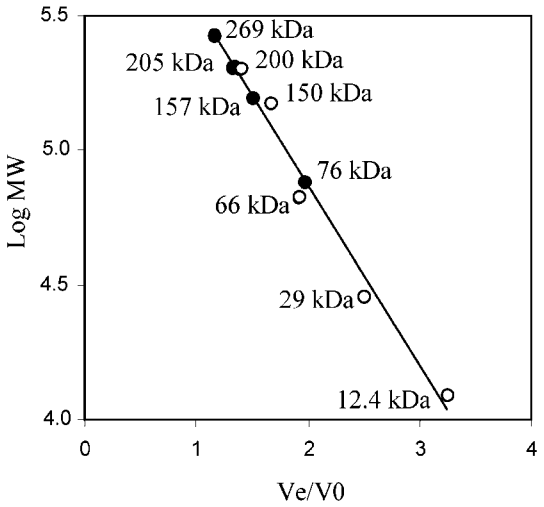

Figure 5. Estimation of the molecular mass of the sorghum LOR (●) of a partially purified extract on a gel filtration Superose 12 HR column. Calibration curve made using standard molecular mass protein markers (○): catalase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). The void volume was determined using blue dextran (2000 kDa).

enzymes from other plant species. For instance, the maize enzymes were coeluted with 200 mM KCl (*27*), whereas in rice, 160 mM KCl was used for the elution of LOR and SDH (*30*).

The ratio of LOR/SDH activity obtained for sorghum varied considerably from approximately 0.2 to 5 among the different experiments carried out and depending on the purification procedure used, whereas in rice and maize, LOR/SDH ratios of 1.2 and 4, respectively, were observed (*27*, *30*). These results suggest differential levels of stability or activity of LOR and SDH among these species.

Although LOR and SDH activities were only tested after ammonium sulfate and anion exchange chromatography steps, both enzymes copurified as has been observed for other plant species (see ref *6* for a review), indicating that in sorghum, LOR and SDH may also be present as a bifunctional polypeptide, which agrees with the fact that a bifunctional LOR−SDH protein appears to be a common feature in plants (*6*, *21*). In this study, no evidence was obtained for the presence of monofunctional enzymes, although monofunctional LOR and SDH enzymes have also recently been reported in some plant species and were shown to be differentially expressed and modulated (*22*, *29*, *35*). On the basis of our data, we cannot rule out the existence of LOR and SDH monofunctional enzymes.

Although very high losses of LOR and SDH activities occurred after the anion exchange chromatography step, some samples that maintained relatively high LOR and SDH activities were applied to a gel filtration Superose 12HR 10/30 (Pharmacia) column to estimated the molecular masses of LOR and SDH. The elution profile and enzyme assays revealed four peaks of LOR but not SDH activity, with apparent molecular masses of approximately 270, 205, 157, and 76 kDa in elution order (**Figure 5**). The lack of SDH activity could suggest that the peaks represent monofunctional LOR enzymes. However, the combined activity of all fractions suggests a further loss in activity, a higher proportion of which could have been by SDH. In contrast, Kemper et al. (*33*) separated the LOR and SDH polypeptides by elastase digestion and demonstrated little effect of proteolysis on SDH activity but did show inactivation of LOR. Molecular mass determinations of the maize bifunctional native enzyme (dimeric form) indicated an estimated value of

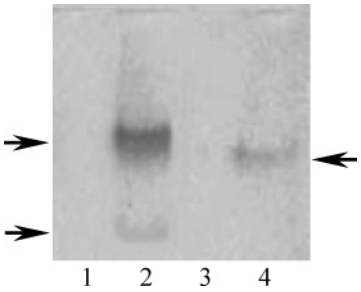

Figure 6. SDH activity staining following nondenaturing PAGE. Lane 1, maize extract stained for SDH activity, but the substrate, saccharopine, was excluded from the reaction mixture; lane 2, the same maize extracted stained for SDH activity but in the presence of saccharopine; lane 3, sorghum extract stained for SDH activity, but the substrate, saccharopine, was excluded from the reaction mixture; lane 4, the same sorghum extracted stained for SDH activity but in the presence of saccharopine. Extracts were isolated from 20 DAP maize seeds and stage 2 sorghum seeds and were partially purified to the DEAE-Sephacel step. The arrows indicate the SDH activity bands.

260 kDa, but monomers of 125 kDa were also observed, which maintained enzyme activity (*43*). Thus, independent of the loss of SDH activity, based on the molecular masses reported previously, the peaks of 270, 205, and 157 kDa are very similar to those of the bifunctional LOR−SDH polypeptides, whereas the 76 kDa peak is likely to be monofunctional LOR.

SDH Activity Staining. SDH activity staining, following nondenaturing PAGE, revealed the presence of only one SDH activity band (**Figure 6**). The band exhibited a different mobility when compared to the maize SDH activity band used as a control, suggesting that the sorghum enzyme is likely to have a smaller molecular mass to that estimated for the maize enzyme. It is possible that other isoenzymes are present in sorghum as discussed earlier; however, they were not detectable by the native PAGE system used. Furthermore, activity staining for LOR, which would have confirmed whether a monofunctional or bifunctional enzyme protein was present in sorghum, has already proven to be very difficult in all other species, even those exhibiting much higher LOR activities (*6*). Several enzyme preparations were used for enzyme activity staining, but only the DEAE-Sephacel concentrated sample allowed the detection of some SDH activity in the gels. In other plant species such as rice (*30*, *34*), coix (*31*), and maize (*20*, *27*), partially purified extracts obtained by ammonium sulfate precipitation allowed the detection of SDH activity on the gels. Following activity staining, up to five bands of SDH have been identified in rice seeds (*34*). In maize, Azevedo et al. (*20*) reported the presence of two bands of SDH activity, in contrast to one major SDH band observed previously for maize in a different study (*27*) as well as for coix (*31*). The nondenaturing PAGE system is a powerful technique and with better enzyme preparations should be used in future studies to analyze the nature of the LOR and SDH sorghum enzymes.

Amino Acid, Protein, and Enzyme Activity Correlations. The soluble and protein amino acid content of the sorghum seeds was determined by colorimetric procedures during the three stages of development (**Figure 7**). The protein amino acid contents were higher in the early and later stages of seed development, the latter probably indicating the deposition of amino acids into storage proteins in the later stages of the seed maturation process, while the soluble amino acids showed the highest concentration at stage 2, probably the period of the maximum rate of protein synthesis.

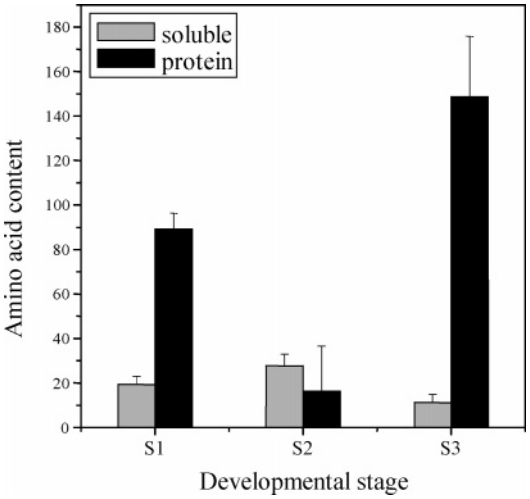

Figure 7. Total soluble and protein amino acid content (μmol g^{-1} fresh seed mass) from developing sorghum seeds at different developing stages. S1, early development stage (93 days post-emergence); S2, intermediary stage (97 days post-emergence—milk stage); and S3, later development stage (101 days post-emergence).

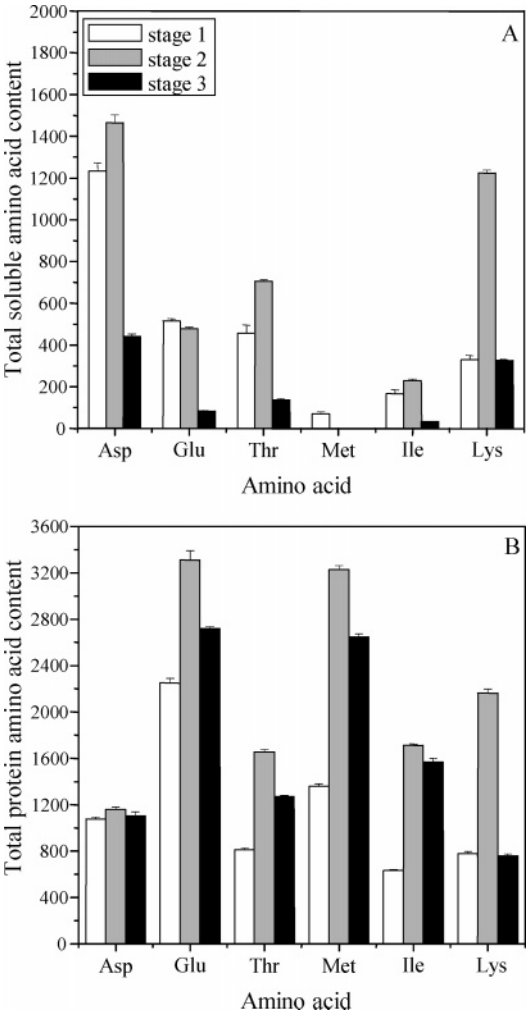

Figure 8. Soluble (**A**) and protein (**B**) amino acids content (nmol g^{-1} fresh seed mass) in developing seeds at three developmental stages.

Both the soluble and the protein lysine concentrations were relatively high (**Figure 8**), when compared to the values reported for other cereal crops (*18*−*20*, *30*, *37*, *41*). At stage 2, the protein lysine concentration was almost 2-fold higher than the soluble concentration, indicating a high rate of lysine incorporation into the storage proteins synthesized. However, high soluble lysine does not necessarily correlate with a higher production of high-lysine proteins since in high-lysine rice mutants the total soluble amino acids pool was 75% when compared to the wild-type, but soluble lysine, methionine, phenylalanine, and proline were considerably lower in the mutant seed (*44*). It was also suggested that later in the development of rice seeds an enhanced lysine accumulation occurred, suggesting an extension of the temporal sequence of events during which lysine-rich proteins are being synthesized and packaged (*44*). Amino acids such as aspartic acid, lysine, threonine, and methionine are probably incorporated rapidly into specific classes of storage proteins. In addition, results reported by Shaefer and Sharpe (*44*) also suggest a higher rate of synthesis of these lysine-rich proteins as well as a reduction in the rate of catabolic activity in the high-lysine rice mutants when compared to the wild-type. In barley, the lysine-rich fraction (albumins and globulins) is synthesized at a faster rate during early stages of seed formation, whereas lysine-poor storage proteins (such as the prolamins) dominate later stages of protein synthesis (*45*, *46*). Glutelins, which exhibit intermediate lysine content, increase linearly with time during seed development. Thus, if a similar mechanism it assumed to occur in sorghum seeds, the low activities of LOR and SDH observed in this study may result in excess lysine for incorporation into lysine-rich proteins.

The relative concentrations of soluble aspartic acid (precursor of lysine biosynthesis), threonine, isoleucine, methionine, and lysine during the later seed development stages suggest that carbon molecules may be being driven to the lysine branch of the aspartate pathway since the ratios observed for lysine, particularly in the last two stages, are higher than for the other aspartate derived amino acids. This is particularly important since it indicates that there may be an altered regulation of the key enzymes controlling the distinct branches of the pathway, which can be confirmed in the future by a complete analysis of all enzymes, including AK, HSDH, DHDPS, and TS, in both wild-type and high-lysine sorghum mutants.

The results of amino acid analysis for Massa 03 (**Figure 8**) and the infrared measurements (**Figure 9**) clearly indicate that the commercial genotype Massa 03 used for the isolation of LOR and SDH has a high lysine concentration since the

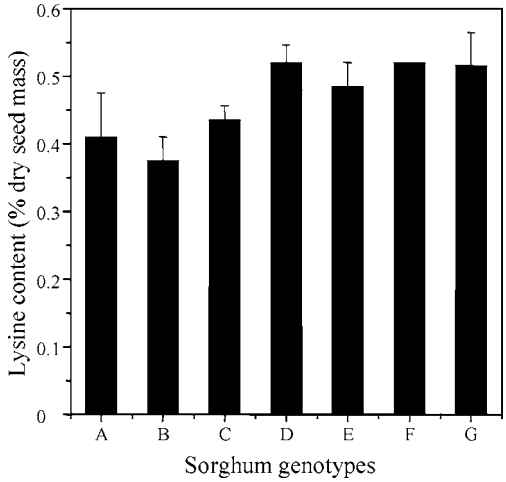

Figure 9. Lysine content of mature seeds of several sorghum mutants (ICRISAT accessions) and Massa 03 (Dow Agroscience) determined by infrared absorbance transmission. (**A**) IS 11758; (**B**) IS 11167; (**C**) IS 16210; (**D**) IS 10477; (**E**) IS 22204; (**F**) IS 5603; and (**G**) Massa 03. The results are expressed on % of dry seed mass.

concentration is in the same range as the high-lysine mutants analyzed. This result could explain at least in part the reduced LOR and SDH activities, which characterized this sorghum line during isolation and purification, in a manner similar to the maize high-lysine mutants, such as the opaque-2, which exhibit higher lysine content due to a drastic reduction in lysine catabolism and altered distribution of storage protein (*18*−*20*). To confirm such a possibility, LOR and SDH activities were also determined during sorghum development and in rice seeds (milk stage, which accounts for the highest LOR and SDH activities) (**Figure 2**). The results indicate that both sorghum enzymes exhibited very low activities when compared to the activity in rice immature seeds. Gaziola et al. (*30*) isolated and purified LOR and SDH from the developing seeds of rice and showed that the activities are reduced when compared to other cereal crops analyzed, which could partially explain the naturally higher lysine concentration normally observed in rice when compared to other cereal crops. In a similar manner, the maize opaque-2 mutant and derived quality protein maize (QPM) varieties also exhibited drastic reductions in lysine degradation when compared to wild-type maize lines also leading to a higher lysine concentration in the seed (*37*, *43*). Several other high-lysine maize mutants also exhibited reduced LOR and SDH activities (*18*−*20*).

As far as legume species are concerned, the high-lysine concentrations have also been explained, at least in part, by the reduced rate of lysine catabolism (*41*). Therefore, the results obtained in this research confirm several previous reports suggesting that a reduction in the rate of lysine catabolism is essential for lysine accumulation in seeds. They also emphasize that the genetic manipulation of crops for the high-lysine trait should consider the degradation pathway, which appears to be as important as lysine synthesis, especially for the seeds as previously suggested (*23*), which directly affect the soluble and total lysine concentrations. Although the available literature strongly indicates that a high-lysine concentration in seeds is inversely correlated to the rate of degradation, sorghum may be a plant that regulates LOR and SDH activity regardless of the lysine content of the seed. Such a hypothesis can only be tested by a side-by-side comparison of the enzyme activities in low versus high lysine sorghum varieties. Such an analysis is part of an ongoing project in our laboratories, which also involves the study of storage proteins by SDS and 2-D PAGE. We have recently shown that the concentration of the storage protein kafirin (sorghum prolamin) in Massa 03 is low when compared to other high-lysine sorghum mutants and wild-type varieties, in a manner similar to the opaque-2 maize mutant (unpublished results).

There is a good nutritional potential for Massa 03, particularly due to the high lysine content observed in the seeds. Furthermore, apart from the high yields that can be obtained with sorghum when compared to other cereal crops, the storage protein and amino acid content variability should be considered in further breeding programs to contribute to a better understanding of amino acid metabolism.

ABBREVIATIONS USED

AK, aspartate kinase; DHDPS, dihydrodipicolinate synthase; HSDH, homoserine dehydrogenase; LOR, lysine 2-oxoglutarate reductase; OPA, *o*-phthaldialdehyde; SDH, saccharopine dehydrogenase; TS, threonine synthase.

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